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Glucose, Mannose, and Fructose Metabolism by Ascites Tumor Cells:
Effects of Dinitrocresol. (21185)

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The present experiments are part of a program dealing with the metabolism of ascites tumor cells derived from the Ehrlich carcinoma(1) or from sarcoma 180. Such tumor cells are now under wide investigation because of the potential advantages which they offer over tumor slices or homogenates(2-6). The relative rates of utilization of glucose, mannose, and fructose have been measured, with and without addition of different concentrations of 4,6-dinitro-o-cresol (DNC).

Methods. Ehrlich carcinoma. The ascites tumor was maintained in male or female mice of the Cox strain. On the 7th to the 9th day after inoculation, each mouse was anesthetized with ether or chloroform and the ascites fluid aspirated, without anticoagulant, into a pre-chilled receptacle; the fluid from 12 mice was pooled. The crude ascites fluid was diluted with 4 volumes of a cold solution (Solution A) of the following composition: NaCl, 0.14 M; KCl, 0.006 M; CaCl₂, 0.003 M; MgCl₂, 0.001 M; glycylglycine, 0.025 M; pH adjusted to 7.4. The diluted cell suspension was filtered through 2 layers of cheesecloth and centrifuged lightly in tared centrifuge tubes (Sorvall medium centrifuge, Model SP/X) by bringing the speed to 1300 R.P.M. over a period of 3 minutes and then shutting off the power. The supernatant fluid was drawn off and discarded, removing most of the red and white blood cells and some unsedimented

ascites cells. The sedimented cells were re-suspended in the same solution, using a volume 5 times that of the original ascites fluid. The suspension was centrifuged as before and the supernatant fluid discarded. The sedimented ascites cells were resuspended in Solution A, making the total volume twice that of the original ascites fluid; the ascites cells were then collected in a packed layer by bringing the speed to 2500 R.P.M. over a period of 5 minutes and shutting off the power. The weight of the packed cells was ascertained by weighing in the tared centrifuge tubes. The weighed cells were finally suspended in Solution A to give a concentration of 250 mg wet cells per cc of total suspension.

Anaerobic consumption of glucose, mannose, and fructose by the ascites tumor cells was substantially larger in isotonic NaCl or a balanced salt solution than in isotonic KCl.

The experiments were carried out in Warburg flasks: main compartment, 0.3 cc glycylglycine buffer (0.25 M), 0.1 cc KHCO₃ (0.02 M), 1 cc hexose solution to give the concentration desired,* 0.6 cc solution of 4,6-dinitro-o-cresol (DNC) to give the final concentrations shown; side arm: 1 cc cell suspension; center cup, 0.3 cc 5 N NaOH

* Hexose concentrations were 5-10 millimolar. Rates of utilization were not increased by using higher substrate concentrations.

TABLE I. Glucose and Fructose Used and Lactate Formed by 250 mg Wet Weight Washed Ascites Tumor Cells (Ehrlich Carcinoma and Sarcoma 180). Results are expressed in micromoles. Temp. 25°C.

Incuba- tion time, min.	Molar conc. DNC × 10 ⁻⁵	Ehrlich ascites carcinoma				Ascites sarcoma 180			
		Glucose substrate		Fructose substrate		Glucose substrate		Fructose substrate	
		Glucose uptake	Lactate formed	Fructose uptake	Lactate formed	Glucose uptake	Lactate formed	Fructose uptake	Lactate formed
Under aerobic conditions									
10	0	2.7	8.2	2.3	8.0	0	6.6	.3	5.1
"	1.6	7.7	14.9	3.3	7.5	5.7	10.6	-1.0	5.0
"	3.2	10.5	16.4	2.3	7.7	9.0	15.0	-.7	5.0
20	0	4.3	11.7	5.0	9.8	4.3	8.6	2.3	5.8
"	1.6	9.3	21.7	4.5	11.2	11.0	16.3	1.7	6.8
"	3.2	11.3	25.4	5.0	9.8	18.7	22.2	5.3	6.8
40	0	5.3	15.8	5.0	13.5	4.7	10.9	4.0	7.6
"	1.6	15.1	31.0	5.7	15.3	14.7	24.8	5.3	9.9
"	3.2	21.3	31.9	8.7	16.7	22.6	28.3	6.7	10.4
Under anaerobic conditions									
10	0	5.5	11.2	1.0	7.5	3.0	9.1	.9	7.1
"	1.6	6.0	13.0	1.5	6.9				
"	3.2	8.3	14.6	2.8	7.4	3.7	11.1	1.3	6.0
20	0	8.7	16.5	2.2	11.2	6.2	14.4	3.5	10.2
"	1.6	11.7	23.0	3.8	10.3				
"	3.2	12.0	24.9	3.0	11.1	7.7	18.9	2.7	10.5
40	0	12.7	25.8	6.5	18.7	9.4	23.9	7.5	16.2
"	1.6	18.3	32.2	6.2	18.0				
"	3.2	19.7	33.5	6.8	18.2	12.0	29.9	6.7	15.5
Initial conc., μM: glucose aerobic Carcinoma Sarcoma fructose aerobic Carcinoma Sarcoma									

(for aerobic experiments only); gas phase, air or nitrogen. Up to this point all procedures were conducted at 5°C. The flasks were shaken at 25°C, gassed, closed, equilibrated for 10 minutes. The cells were tipped in from side arm and observations begun. After the desired incubation the flasks were transferred to an ice bath and the following aliquots removed: 1 cc for lactate analysis(7); 1 cc for hexose determination(8); the remainder for determination of final pH. Suitable zero time samples were prepared and handled similarly without incubation.

Sarcoma 180. The original inoculum of Crocker sarcoma 180 in the ascites form was obtained by courtesy of Dr. Kanematsu Sugiura, of the Sloan-Kettering Institute. The tumor was maintained in male or female Sutter Swiss mice, the ascites cells being obtained from each mouse on the 7th day after inoculation. These ascites cells were obtained and handled throughout by exactly

the same procedures as were used for the Ehrlich ascites cells.

Results. Hexose consumption and lactate formation for 3 time periods, with glucose or fructose as substrate, are shown in Table I. The total uptake of glucose, particularly for the longer periods of time and under the stimulating influence of dinitroresol, was much higher than that of fructose. It should also be noted that under both aerobic and anaerobic conditions the uptake of glucose and the lactate production therefrom were markedly increased by addition of dinitroresol, while relatively little effect was exerted by the same agent on the uptake of fructose and the production of lactic acid therefrom. The rates of hexose consumption and lactate formation with glucose, mannose, or fructose as substrate, without and with the addition of 7 concentrations of dinitroresol, are shown for the Ehrlich ascites carcinoma cells in Fig. 1 and for the ascites sarcoma cells in Fig. 2. The values for glucose were obtained with a

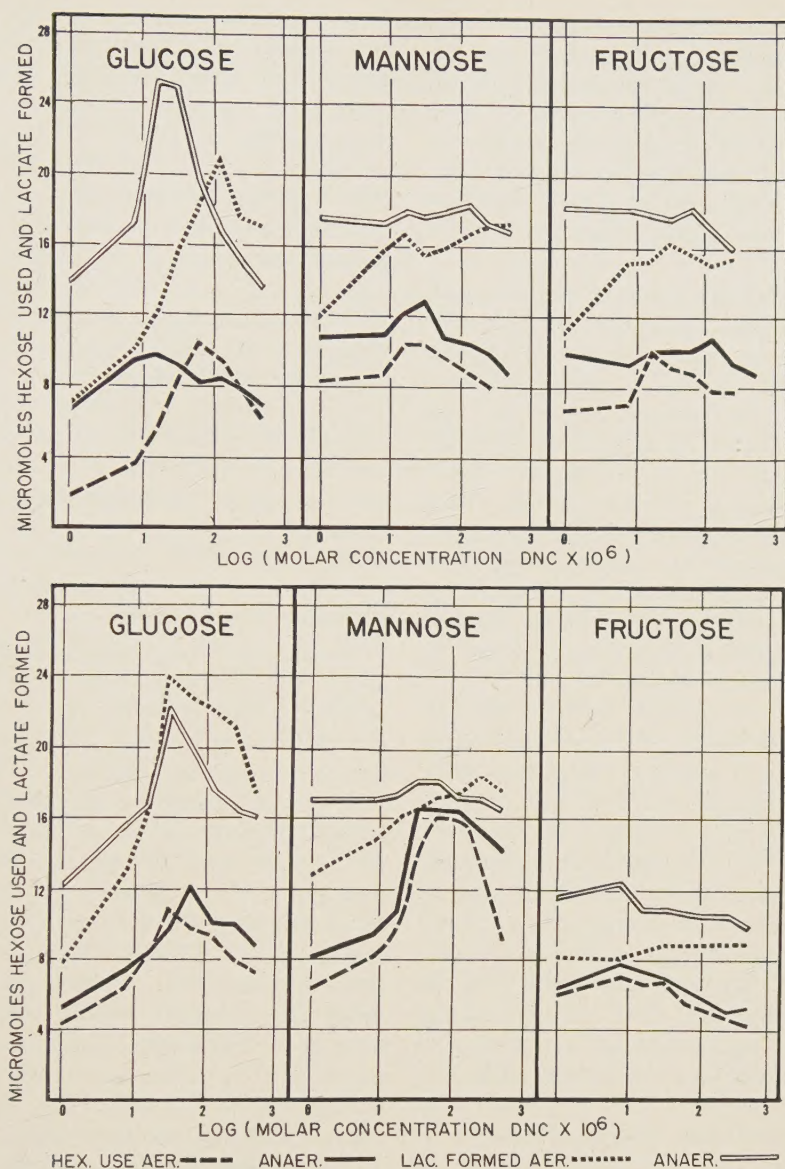


FIG. 1 (top). Hexose uptake and lactate production by Ehrlich ascites carcinoma cells under aerobic and anaerobic conditions and effect exerted by different concentrations of 4,6-dinitro-o-cresol (DNC). Results for glucose were obtained with a 10-min. period of incubation, those for mannose and fructose with a 40-min. period, these being the maximum time periods which could be employed without exhaustion of hexose. Initial hexose concentrations were: glucose, 13.2 μ M; mannose, 28.9 μ M; fructose, 30.4 μ M. Incubation at 25°C.

FIG. 2 (bottom). Hexose uptake and lactate production by ascites sarcoma 180 cells. Experiments were run and recorded as described for Fig. 1. Initial hexose concentrations were: glucose, 14.3 μ M; mannose, 26.0 μ M; fructose, 30.4 μ M.

10-minute period of incubation; those for mannose and fructose with a 40-minute period of incubation, preliminary experiments having been made to establish these as the maximum time periods which could be employed without

decrease in rate due to exhaustion of hexose. Utilization of glucose per hour anaerobically for both the carcinoma and sarcoma cells was over twice that for fructose.

Neither type of tumor cell produced any

lactate, either aerobically or anaerobically, with or without DNC, when used without addition of hexose. From further experiments it was found that both types of tumor cell were incapable of taking up galactose, arabinose,[†] or xylose, either aerobically or anaerobically, with or without the addition of DNC, and that no lactic acid was produced from any of these sugars.

With the carcinoma, and still more markedly with the sarcoma, the aerobic and anaerobic uptake of glucose was increased by addition of dinitrocresol, reached a peak, and subsequently fell; furthermore, the aerobic and anaerobic production of lactate rose markedly with increasing concentrations of DNC, reached a peak, and subsequently fell. With glucose as substrate, the number of micromoles of lactate produced was approximately twice the number of micromoles of glucose taken up. There was marked poisoning of the Pasteur effect, confirming and extending previous observations on tumor slices(9-11).

The uptake of mannose by both the carcinoma and sarcoma cells appeared, under both aerobic and anerobic conditions, to be increased by addition of DNC, with a subsequent fall at higher concentrations. However, precise values for mannose consumption cannot be given because the method of analysis is not completely satisfactory under the present experimental conditions. The lactate production from mannose aerobically was increased by addition of DNC and did not appear to suffer any diminution at higher concentrations of DNC. The lactate production anaerobically, which started at a high level, showed no appreciable increase under the influence of DNC.

The consumption of fructose was not significantly increased by DNC. The lactate production aerobically in the carcinoma series increased under the influence of DNC and did not fall at high concentrations. The anaerobic lactate production from fructose

showed the same effect as was observed in the case of mannose, a fairly high production of lactate in the absence of DNC, which was not appreciably changed on addition of various concentrations of DNC. The oxygen consumption of the tumor cells was increased with increasing concentrations of DNC to a maximum and then decreased to about the original level, even in the absence of added substrate. The addition of glucose had no effect and that of mannose had only a slight effect upon the rate of oxygen consumption at any dinitrocresol concentration; addition of fructose permitted a somewhat higher oxygen consumption, particularly at the optimal concentration of DNC (Fig. 3). It will be noted that the fraction of hexose disappearance accounted for by oxygen consumption is very small, in agreement with the fact that the lactate production usually accounts for nearly all hexose disappearance.

Discussion. The present findings regarding hexose use by ascites cells are of particular interest in two respects. First, the relative rates of glucose, fructose, and mannose utilization differ markedly from those to be expected from current studies on hexokinases of other animal and plant cells; in the present instance the relative rates for glucose, fructose, and mannose are approximately 2:1:1, respectively, as compared to 1:1.4:0.5 for yeast or brain hexokinase(12,13), and 1:1.8:1.2 for *Arbacia* egg hexokinase(14). Further studies are required to establish whether the type and amount of hexokinase in ascites cells can account for these differences or whether the use of hexoses is determined by their relative rates of entrance into these cells.

The second finding to be stressed is the difference in the effect of DNC upon anaerobic utilization of glucose or mannose on the one hand, and of fructose on the other. The stimulation of anaerobic glucose consumption by DNC here observed is one of the largest obtained with any animal or plant cell (See Simon(15) for review); anaerobic use of mannose is also markedly stimulated by DNC, particularly in the sarcoma 180 ascites cells. In contrast, anaerobic use of fructose is *not* stimulated by DNC, although the basal rate for fructose is approximately the same as for

[†] Lactate production by the ascites carcinoma cells from 0.01 M glucose was reduced approximately 96, 80, and 50% when arabinose was concurrently present at concentrations of 0.3, 0.2, and 0.15 M, respectively. These results suggest a corresponding diminution of glucose uptake.

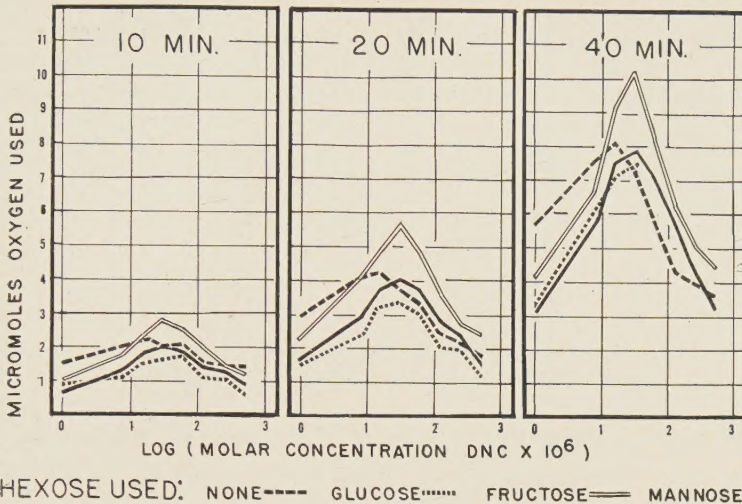


FIG. 3. Oxygen consumed by 250 mg wet wt washed Ehrlich ascites carcinoma cells without addition of hexose and with 10 millimolar glucose, fructose, or mannose. Incubation at 25°C.

mannose. Thus, it would appear that the pathway for utilization of fructose in these cells is not subject to the same regulatory mechanisms as those for glucose and mannose; further experiments to elucidate the nature of this difference are under way.

A third point of some interest is that, whereas the number of micromoles of lactate formed from glucose or fructose is generally approximately twice the number of micromoles of hexose used, lactate production accounts for only about half of the mannose used at optimal concentrations of DNC. This suggests that mannose isomerase may be a limiting factor for conversion of mannose to lactate in these ascites cells.

Summary. 1. Ascites tumor cells derived from Ehrlich carcinoma or sarcoma 180 consume glucose, mannose, or fructose, but not galactose, xylose, or arabinose. Under anaerobic conditions the hexose consumed is approximately accounted for by lactate produced. 2. The consumption of glucose and the production of lactate therefrom under anaerobic conditions are markedly stimulated by dinitroresol. In contrast, the anaerobic consumption of fructose and of lactate production therefrom and the anaerobic production of lactate from mannose are not stimulated by DNC. 3. The aerobic consumption of glucose and production of lactate therefrom are raised

by DNC to nearly the anaerobic levels; there is thus strong poisoning of the Pasteur effect. The aerobic consumptions of mannose and fructose are less influenced by DNC, but the production of lactate therefrom is raised at higher concentrations of DNC to the anaerobic level. 4. The oxygen consumption of both carcinoma and sarcoma cells, with and without addition of DNC, is virtually unaffected by the presence or absence of assimilable hexoses.

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Renal Hemodynamics During Erect Lordosis in Normal Man and Subjects with Orthostatic Proteinuria.* (21186)

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Orthostatic proteinuria has been attributed to a marked reduction in renal blood flow occurring characteristically in this condition during standing(1-4). The inference that there is a difference in hemodynamic postural response between orthostatic proteinuria patients and normal individuals is based on inadequate evidence chiefly utilizing urea and creatinine clearance methods.

Smith(5) described reduction in renal plasma flow and glomerular filtration rate in a normal subject in the passive erect posture (tilt). The confirmatory data of Brun, Knudsen and Raaschou(6) are of equivocal significance since observations were made during periods of marked change in urinary volume, and some specimens were collected without urethral catheterization. In the present study observations on renal hemodynamics in recumbency and erect lordosis have been made in normal subjects and individuals with orthostatic proteinuria under conditions providing relatively constant rates of urine flow. The findings suggest that there is no significant difference in the renal hemodynamic response of these two groups.

Methods. Glomerular filtration rate (GFR) as measured by inulin clearance and renal plasma flow (RPF) as measured by p-aminohippurate clearance were determined in 5

normal young adult males and 5 with orthostatic proteinuria. Observations were made with subjects in a basal state and under moderately hydropenic conditions. Clearances were determined during three 20-minute control periods with the subject in the supine position and during 15 to 20 minutes in erect lordosis. All urine specimens were obtained by urethral catheterization and analyzed for protein as well as for the clearance substances. Inulin was determined by a modification of Harrison's method(7); p-aminohippurate by the method of Smith and associates(8). Quantitative urinary protein was determined by the biuret method of Foster, Rick and Wolfson(9) in those specimens exhibiting protein with 20% sulfosalicylic acid [>10 mg % (10)].

Results. In the 5 normal individuals (Table I) GFR during lordosis decreased an average of 29%, ranging from -6% to -56%; RPF an average of 37% with a range from -9% to -55%. The filtration fraction was unchanged in 2 and increased in 3. Urinary volume (V) decreased an average of 41% with a range from -7 to -67%. Urinary protein did not appear during or after erect lordosis.

In the 5 subjects with orthostatic proteinuria (Table II) GFR during lordosis decreased an average of 17% with a range of +6% to -40%; RPF an average of 27%, ranging from -11% to -42%. The filtration fraction was unchanged in 2 and increased in

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TABLE I. Effect of Erect Lordosis on Renal Hemodynamics, Urine Volume and Protein Excretion in 5 Normal Subjects. Age 20 to 24 Years.

GFR	RPF		Filtration fraction		Urine vol (V)		Protein excr.		% change		
Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent, mg/min.	Erect lordosis, mg/min.	GFR	RPF	F.F.	V.
114	77	597	.19	.22	1.41	.91	0	-32	-41	+16	-35
112	98	545	.21	.27	1.16	.73	0	-12	-33	+29	-37
140	131	545	.26	.26	1.42	1.32	0	-6	-9	0	-7
140	86	748	.19	.22	1.26	.52	0	-39	-47	+16	-59
109	48	728	.15	.15	2.56	.84	0	-56	-55	0	-67

* Avg of 3 periods.

TABLE II. Effect of Erect Lordosis on Renal Hemodynamics, Urine Volume and Protein Excretion in 5 Subjects with Orthostatic Proteinuria. Age 18 to 24 years.

GFR	RPF		Filtration fraction		Urine vol (V)		Protein excr.		% change		
Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent,* cc/min.	Erect lordosis, cc/min.	Recumbent, mg/min.	Erect lordosis, mg/min.	GFR	RPF	F.F.	V.
100	60	502	.20	.18	.69	.26	0	-40	-32	-10	-62
134	112	899	.15	.21	1.34	1.48	0	-16	-42	+40	+10
111	101	591	.19	.22	.52	.52	0	-9	-20	+16	-27
137	98	634	.22	.23	.87	.57	0	-28	-32	+5	-34
91	96	613	.15	.18	.62	.74	0	+6	-11	+20	+19

* Avg of 3 periods.

3. Urinary volume decreased in 3, increased in one and remained unchanged in one. Proteinuria averaging 0.61 mg/min. appeared during erect lordosis in these subjects.

Discussion. These results indicate that significant reduction in glomerular filtration rate and renal plasma flow, usually with an increased filtration fraction occurs in the erect lordotic posture in both normal individuals and those with orthostatic proteinuria. The changes in these two groups appear to be in the same range in the 10 subjects studied here. In the patients with orthostatic proteinuria the rate of protein excretion is not related to the degree of concurrent renal hemodynamic change on erect lordosis. These observations do not confirm any direct or causal relationship between alterations of renal hemodynamics in erect lordosis and the excretion of protein as suggested by others(1-4).

Despite a moderate hydropenic state with control urinary volumes averaging 1.20 cc/min, the anticipated antidiuresis of the erect posture was obtained in 7 of the 10 subjects.

Errors in clearance determinations inherent in studies by others(4,6), due to transition from induced water diuresis during recumbency to oliguria on standing were avoided in the present study. The hydropenic state assured initial low urine flow, preventing extreme change in urine volume on assumption

of erect lordosis. Precise urine collections were obtained by urethral catheterization.

Conclusions. 1. Decreased glomerular filtration rate, renal plasma flow and urine volume of a comparable degree occur in erect lordosis in both normal man and subjects with orthostatic proteinuria. 2. The rate of protein excretion in orthostatic proteinuria is not related to the magnitude of hemodynamic change occurring with erect lordosis.

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Inhibition of Egg-White Edema by Proteolytic Enzymes. (21187)

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Trypsin has a marked anti-inflammatory action(1,2) and a thrombolytic effect(2), and catalyzes the conversion of profibrinolysin to fibrinolysin(3). The action of trypsin on inflammation occurs before its effect on thrombi(2). The mechanism of the anti-inflammatory effect is not clear but its rapidity appears to rule out activation of another enzyme. The following results indicate that the profibrinolysin-fibrinolysin reaction is not of primary importance in this action.

Methods. A number of proteolytic enzymes were tested to determine their effect on the inflammatory reaction. The egg-white edema reaction of Selye(4), as previously reported(1), was used throughout. This has been used as a standard testing method in our laboratory, although trypsin has been shown to reduce edemas(5) produced by other agents, such as yeast(6). The egg-white edema test was carried out on rats 60-90 g in weight. The enzymes tested were injected

TABLE I. Inhibition of Egg-White Edema by Proteolytic Enzymes.

Enzyme	Dose (mg/kg)	Wt difference, rt. leg-left leg (g)	Stand. dev.	Inhibition (%)
C*	—	1.50	.06	—
Trypsin	10	.80	.18	45
C	—	1.38	.08	—
Trypsin	2	.90	.05	35
C	—	1.48	.07	—
Trypsin	1	1.40	.18	0
C	—	1.68	.13	—
Streptokinase†	20	1.30	.12	20
C	—	1.68	.13	—
Streptokinase	10	1.40	.12	15
C	—	1.63	.23	—
Streptokinase	2	1.50	.27	0
C	—	1.90	.09	—
Chymotrypsin	2	.93	.20	50
C	—	1.90	.09	—
Chymotrypsin	1	1.83	.20	0
C	—	1.68	.13	—
Prolase B	10	1.13	.15	30
C	—	1.90	.09	—
Prolase B	2	1.51	.19	20
C	—	1.90	.09	—
Prolase B	1	1.75	.21	0
C	—	1.97	.17	—
Fibrinolysin‡	400	1.70	.12	15
C	—	1.93	.08	—
Fibrinolysin	200	1.93	.11	0

* Control.

† The commercial preparation "Varidase" was used.

‡ Obtained by courtesy of Dr. E. C. Loomis, Parke, Davis & Co.

subcutaneously on the ventral surface in 0.3 ml of aqueous solution and again 30 minutes later. Immediately after the second injection 0.1 ml of egg-white was injected subcutaneously into the dorsal side of the right hind paw and 0.1 ml of saline into the left. After 90 minutes the animals were killed with ether. Both hind legs were removed at the knee joint and weighed. The difference in weight between the saline-injected and egg-white injected legs was taken as an index of the extent of edema. A decrease in weight gain in the edematous leg over that of controls indicates inhibition of edema formation.

Results are presented in Table I. Doses of the various enzymes used are expressed by weight, since unitages are not interconvertible. Figures refer to total dose, given in 2 portions as noted above. The differences in weight between the right (egg-white injected) and left (saline-injected) legs are averages for 5 ani-

mals. Since the extent of edema obtained varied to some extent with the particular group of animals and with the source of egg-white each experimental group is compared to its own control run simultaneously.

The results show that all the enzymes tested were effective in inhibiting edema formation. However, the effects appear to bear no relationship to ability to catalyze the reaction profibrinolysin-fibrinolysin. Thus chymotrypsin, which does not catalyze this reaction(3), is of the same order of activity as trypsin; the effect of fibrinolysin even at the very high doses used is of doubtful significance. The fact that an effect was shown by several different enzymes suggested that it might be a function of simple proteolytic activity. In order to investigate this partially and completely inactivated samples of trypsin were tested for their effect on egg-white edema. Proteolytic activity was determined by the method of Anson(7).

For standard crystalline trypsin, at 100% activity, the minimum effective dose was 2 mg/kg (see Table I). For trypsin at 50% activity, the MED was 4 mg/kg; completely inactivated trypsin showed no anti-inflammatory effect in doses up to 10 mg/kg. This shows definitely that the anti-inflammatory effect of trypsin is a function of its proteolytic activity. Although the profibrinolysin-fibrinolysin system is not precluded, these results show that anti-inflammatory effects are manifested by enzymes which do not catalyze this reaction. Moreover fibrinolysin itself has a comparatively weak action. The anti-inflammatory effect of proteolytic enzymes may bear some relation to the work of Spector(8) which indicates that polypeptides are involved in the production of inflammation.

Summary. In an attempt to elucidate the mechanism of the anti-inflammatory action of trypsin a number of proteolytic enzymes were tested against edema produced in rats by local injection of egg-white. Enzymes tested included chymotrypsin, streptokinase, prolase B and fibrinolysin. All were active in suppressing the experimental edema and all showed the same order of activity as trypsin except fibrinolysin, which was appreciably weaker.

In the case of trypsin the anti-inflammatory action was found to be a function of proteolytic activity; it varied with the activity of partially and completely inactivated samples of this enzyme.

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Use of Artificial Kidney for Removal of Barbiturates in Dogs.* (21188)

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Barbiturates are responsible for one-third of all fatalities resulting from exposure to toxic substances and for 30 to 40% of all hospital admissions due to ingestion of poisons(1,2). Prompt recognition of barbiturate intoxication coupled with effective therapy can be life saving. With the advent of ultraviolet spectrophotometric methods(3-7), much of the difficulty previously involved in the laboratory determination of barbiturates in biological fluids has been eliminated. In 1 to 2 hours, one can not only determine the barbiturate level in biological material but also, in many cases, the specific barbiturate that was ingested. Thus, prompt chemical confirmation of barbiturate intoxication is now possible.

The removal of toxic substances, such as salicylates and bromides, by the use of hemodialysis has already been demonstrated(8, 9). There are relatively few available data to indicate the feasibility of this technic for the removal of barbiturates, although treatment of several patients has been reported(10). In a preliminary report from this laboratory hemodialysis was shown to be of limited value in pentobarbital intoxication in dogs(11).

Methods. A series of dogs was given a known dose of a specific barbiturate intravenously or intraperitoneally. Using Goldbaum's technic(3) blood or plasma barbiturate levels were determined at 2-hour intervals, until 12 hours elapsed, then at 6-hour intervals until the animal recovered or died. Interspersed with the "control" dogs, another group of dogs, referred to hereafter as "dialyzed" dogs, were given the same dose of barbiturate and treated by hemodialysis 1 to 3 hours later. A Skeggs-Leonards(12) artificial kidney with a

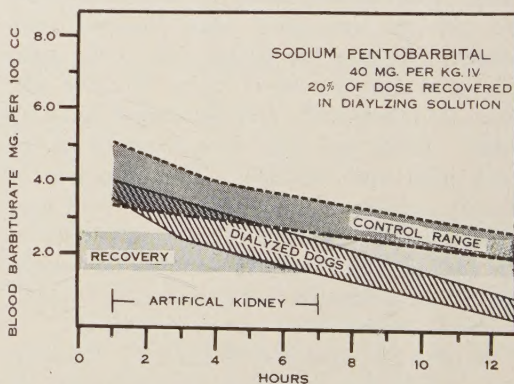


FIG. 1. Blood pentobarbital levels as a function of time. Control and dialyzed ranges represent all the values between maximum and minimum blood pentobarbital levels for all dogs in the respective groups. Recovery range represents spread of blood pentobarbital levels obtained when animals first began to move.

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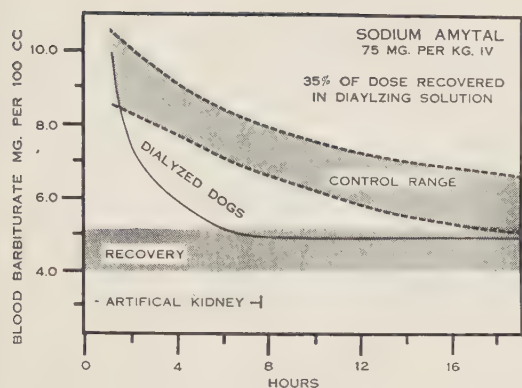


FIG. 2. Blood amobarbital levels as a function of time. Since all blood amobarbital levels for "dialyzed" dogs were so similar, the single line represents average value.

cellophane dialysis area of 20000 sq cm. operating with a blood flow of 200 ml/min. was used. The dialyzing solution had an electrolyte composition corresponding to the extracellular fluid of dogs(12). Generally, 200 to 300 litres of this dialyzing solution were used for each animal during the 4- to 8-hour dialysis period. The dog received no other therapy. In addition to the blood barbiturate levels, the amount of barbiturate in the total dialysate was also determined. The amount of barbiturate that can be removed by hemodialysis is a function of the "free" barbiturate, which is not bound to plasma proteins. Protein binding of various barbiturates was determined in some of the dogs by putting one litre of sterile dialyzing solution(12) in the dog's peritoneal cavity. The ratio of the barbiturate concentration in the peritoneal fluid and blood

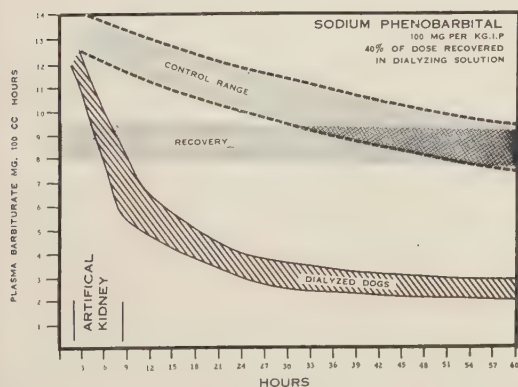


FIG. 3. Plasma phenobarbital levels as a function of time (100 mg/kg).

was determined at 4-hour intervals. After 12 hours, this ratio became constant and represented the percentage of "free" barbiturate.

Results. Sodium pentobarbital 40 mg/kg intravenously (Fig. 1). Three of the 8 "control" animals died as did 2 of 7 "dialyzed" dogs. Fig. 1 indicates that the "dialyzed" dogs began to move 5 hours sooner than the "controls". All the surviving animals completely recovered within approximately 22 hours after the original injection of the drug.

Amobarbital 75 mg/kg intravenously (Fig. 2). This amount of amobarbital administered intravenously over a 5- to 10-minute period is so close to the lethal dose that of the 15 dogs so injected, 7 died within 15 minutes. Of the 8 survivors, 5 were not treated. These "control" dogs were not asymptomatic until 36 to 48 hours had elapsed. One "control" dog died

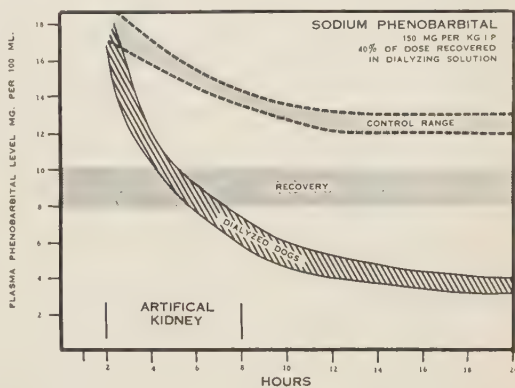


FIG. 4. Plasma phenobarbital levels as a function of time (150 mg/kg).

between 12 to 24 hours after the initial injection.

In the remaining 3 dogs, hemodialysis was started one hour after the initial injection of amobarbital and continued for 6 hours. By 19 hours, 2 dogs were completely recovered and the other recovered 28 hours after the initial injection.

Phenobarbital 100 mg/kg intraperitoneally (Fig. 3). Nine dogs were used in this series; 4 were controls and 5 were treated. All the dogs survived but the "control" dogs were not asymptomatic until 72 to 114 hours after the initial injection. In the 5 treated animals the plasma phenobarbital level dropped very sharply after hemodialysis was started. By

TABLE I. Renal and Artificial Kidney Barbiturate Clearances.

	Renal clearance (cc/min.)	Artificial kidney clearance (cc/min.)
Pentobarbital	0.7-1.7	15-26
Amobarbital	2.6-5.2	23-29
Phenobarbital	*	33-43

* Not analyzed. Barbitol is not bound to plasma proteins. Since renal clearance of barbitol in the dog is 6 cc/min.(14), one would expect the phenobarbital clearance to be somewhat less than this figure.

24 hours, 4 were standing and the last animal (heaviest) recovered completely by 72 hours.

Phenobarbital 150 mg/kg intraperitoneally (Fig. 4). Eleven dogs were used in this series, 5 were controls and 6 were treated. Four of the "control" dogs died at 6, 20, 70 and 72 hours respectively. Plasma phenobarbital levels at the time of death were all greater than 14 mg/100 ml. The surviving dog (13 kg) was completely anaesthetized for 3 days and could barely walk after 6 days. All the treated animals survived and began to move after 8 hours of hemodialysis. Four were asymptomatic after 24 to 30 hours, the other in 50 hours.

Protein binding. Experiments devised to determine the protein binding of the barbiturates in plasma indicate that 20 to 30% of the total phenobarbital is bound. In the case of pentobarbital, this value is 35% and for amobarbital it is 50 to 60%. Using another technique, Goldman(13), reported the protein binding of these barbiturates to be 20%, 37% and 37% respectively.

Clearance determinations. From the data obtained in these experiments, the renal barbiturate clearances, and the clearances of barbiturates by the artificial kidney, were calculated. No corrections were made for protein binding in the clearance data given in Table I.

Discussion. Kyle(10) used a Kolff-type artificial kidney to treat 3 human patients suffering from barbiturate intoxication. He concluded that "treatment of severe barbiturate poisoning by hemodialysis appears to be practical and effective."

There is no doubt that hemodialysis is a practical form of therapy. However, the effectiveness of this therapy is open to question.

The data presented by Kyle indicate the artificial kidney will remove barbiturates from humans at the rate of 70 to 150 mg/hr. Our animal experiments corroborate this rate of removal. This is to be expected, since the area of the cellophane dialyzing surface is the same in the 2 types of artificial kidney. Thus, under optimum conditions, approximately 7 hours of hemodialysis would be required to remove one g of phenobarbital. The average amount of barbiturate ingested in poisoning incidents is approximately 5 g and in acute cases it may be as high as 10 g. Thus hemodialysis would only remove 10 to 20% of the original dose in a 7-hour period.

Another criterion for the efficacy of hemodialysis in barbiturate intoxication is the clinical response to this therapy. In the case of pentobarbital the "dialyzed" dogs began to move spontaneously approximately 4 hours sooner than the "controls." The rate of decline of blood pentobarbital levels was significantly greater in the "dialyzed" dogs. However, despite these benefits, all the animals took approximately the same time until they became asymptomatic and the relative death rate was the same for both series. It appears thus that hemodialysis is of questionable value in cases of pentobarbital intoxication.

In the case of amobarbital the blood amobarbital level declined rapidly while the animals were on the artificial kidney. Consequently the "dialyzed" dogs began to move spontaneously 10 hours before the "controls" and were completely recovered 17 to 20 hours sooner.

In the case of phenobarbital, there was no question about the beneficial effects of hemodialysis. At the lower dose level employed (100 mg/kg), "control" dogs did not begin to move spontaneously for at least 36 hours, while 4 of the 5 treated animals were able to walk, albeit unsteadily, in 24 hours. At a dose of 150 mg/kg, 4 of the 5 "control" dogs died, while all 6 of the treated animals recovered fully.

In view of these findings, hemodialysis therapy appears to be warranted in the treatment of severe intoxication due to a long-acting barbiturate, such as phenobarbital. The

expected beneficial result should be tempered by the knowledge that the rate of dialysis is slow and that the duration of treatment with the artificial kidney should be longer than the usual 6 hours used in treatment of renal insufficiency. This dialysis period could be shortened considerably if the existing artificial "kidneys" were enlarged to provide more dialyzing area. In the case of intoxication due to short-acting barbiturates, hemodialysis does not seem to be very helpful.

Hemodialysis may be life saving in those cases of acute barbiturate intoxication where the patient is comatose, areflexive with circulatory or respiratory difficulties and where the blood barbiturate level is elevated.

Summary. An artificial kidney of the Skegg-Leonards type was used successfully to lower blood barbiturate levels in dogs. Approximately 15 to 25% of the intravenously injected pentobarbital, 35% of intravenously injected amobarbital, and 40 to 70% of intraperitoneally injected phenobarbital were recovered in the respective dialysates. This therapy was life saving in the phenobarbital experiments, of some value in the amobarbital intoxication, but of questionable value in pentobarbital poisoning.

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Dehydrogenase Activities of Human Platelets.*† (21189)

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Although a good deal is known about the morphology and physical characteristics of platelets, little information is available concerning their biochemical properties. Platelets have been reported to contain certain enzymes such as esterases, acid phosphatase and

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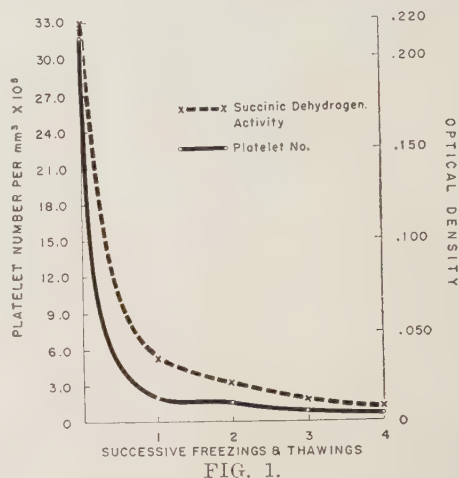
β -glucuronidase(1), but, whereas according to some workers they have little or no intrinsic metabolism(2), others have found that they exhibit a very rapid metabolic activity(3). These activities, however, have usually been observed in freshly prepared platelets and always disappear completely following storage of the specimens for a period of 2 to 3 days (3). There is some indication that the methods used for the collection of blood and for the preparation and storage of platelets may exert an effect on the extent of their observable biochemical properties(4). It is

of interest in this connection that platelets have been stored for as long as one year without significant decrease in cell numbers and that, although morphological changes and loss of clot-retracting ability occur during this time, thromboplastic activity apparently is maintained(2).

Materials and methods. Platelet suspensions were prepared from fresh, versene (ethylenediamine tetraacetate) collected human blood according to the method described by Minor and Burnett(5). Following their concentration the platelets were washed 3 times in physiological saline containing 2% Triton WR-1339[†] and made up in this medium to a standard photometric density (Coleman Junior spectrophotometer wavelength 420 $m\mu$, saline-Triton blank) of 1.301 (5% transmission). This "standard platelet suspension" contained approximately 2.5×10^6 cells per mm^3 . The reduction of 2,3,5-triphenyltetrazolium chloride was used to demonstrate the presence and estimate the levels of the various dehydrogenase systems studied(6). To 1.0 ml volumes of a freshly prepared (4-5 hours after collection) "standard platelet suspension" were added 0.5 ml of 0.20 M substrate, pH 7.0, and 0.5 ml of 0.5% freshly prepared 2,3,5-triphenyltetrazolium chloride in 0.10 M phosphate buffer, pH 7.0, containing 0.1% diphosphopyridine nucleotide. Control tubes were set up to contain instead of substrate either water or physiological saline and, instead of enzyme, saline containing 2% Triton WR-1339. Incubation was allowed to proceed at 37°C for 60 minutes and no measurable decrease in cell number was observed during this period of time. The reaction was stopped by the addition to each tube of 5.0 ml acetone, the mixtures were shaken vigorously and the precipitated cell proteins separated by 10 minutes centrifugation at 1500 r.p.m. The optical densities of the resulting clear supernatants were determined in a Coleman Junior spectrophotometer at a wavelength of 485 $m\mu$ and the various dehydrogenase activities expressed in terms of these optical densities. All determinations were carried out in triplicate and the results reported are the averages.

[†] Rohm and Haas.

Relationship Between Succinic Dehydrogenase Activity and No. of Intact Platelets Remaining After Successive Freezings and Thawings of Standard Platelet Suspension



Cell-free dehydrogenase preparations were obtained (a) by incubating 1.0 ml volumes of a freshly prepared "standard platelet suspension" for 30 minutes at 37°C with 0.05 ml volumes of any one of the following: Toluene, 2% aqueous saponin or 10% aqueous phenol, or (b) by rapidly freezing and thawing a "standard platelet suspension" and repeating this process twice. Both types of treatment were found to result in the lysis of more than 95% of the originally present intact cells. Platelet counts were carried out according to the method described by Rees and Ecker(7). All glassware used in the collection of blood, in the preparation of platelets and during the study of metabolic activities was silicone-coated with Desicote (Beckman).

Results. The following substrates have been investigated: Succinate, l-malate, α -glycerophosphate, lactate, glutamate, β -hydroxybutyrate, glucose, and ethanol. The dehydrogenase systems concerned with the oxidation of all of these substances were found to be actively present in freshly prepared platelets. Omission of either substrate or enzyme (platelets) did not result in the reduction of the dye. Under the particular experimental conditions employed the most active dehydrogenases were those attacking succinate, l-malate, and α -glycerophosphate.

In an attempt to obtain cell-free enzyme

Effect of Cysteine on Dehydrogenase Activities of Human Platelets

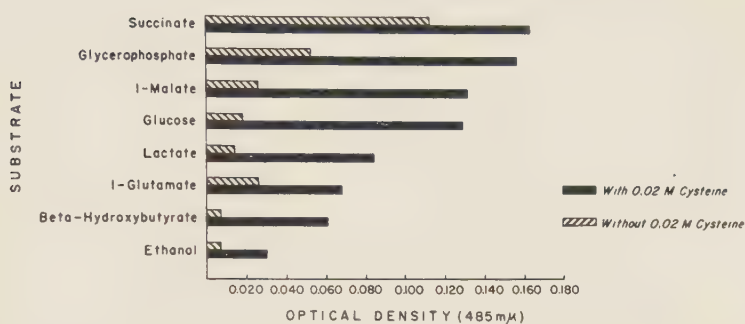


FIG. 2.

preparations it was found that platelets can be lysed quickly and readily by chemicals such as saponin, toluene, and phenol or by the physical means of repeated freezing and thawing. The resulting preparations were found to be homogeneous and opalescent, but completely lacking in dehydrogenase activity. That the activity of these enzymes appears to be closely related to the morphological integrity of the platelets is further substantiated by the data presented in Fig. 1. With increasing cell destruction, as observed following repeated freezing and thawing, the level of succinic dehydrogenase activity falls rapidly and closely parallels the extent of cell destruction. Essentially similar data have been obtained for some of the other dehydrogenase systems.

On the other hand, it was found that the addition to intact, freshly prepared platelets of cysteine in a final concentration of 0.02 M exerts a marked and apparently activating effect on enzyme activity (Fig. 2). The extent of activation varies for the different dehydrogenase systems studied, but appears to be greatest for those exhibiting lower activities in the absence of added cysteine.

Following this initial observation the relationship between cysteine concentration and its ability to activate platelet succinic dehydrogenase was studied. Control experiments were run with cysteine only and, in order to obtain the activation of succinic dehydrogenase proper, the optical density of reduced triphenyltetrazolium chloride as brought about by cysteine oxidation alone was sub-

tracted from that observed in the presence of cysteine and succinate. The extent of activation was found to be most marked up to a cysteine concentration of 5×10^{-4} M, to reach a maximum of 100% at about 25×10^{-4} and to decrease at concentrations above 100×10^{-4} M. Similar observations were made on the relationship between malic and glycerophosphate dehydrogenase activities on the one hand and increasing cysteine concentrations on the other. Here again the rate of activation was found to be maximum up to a cysteine concentration of 5×10^{-4} M and peak levels of 500 and 300% respectively were reached at about 25×10^{-4} M, followed by a marked decrease in activation at concentrations above 100×10^{-4} M.

An essentially similar activating effect on the 3 dehydrogenase systems studied was produced by reduced glutathione, activation again increasing most markedly up to a concentration of about 10×10^{-4} M, reaching a maximum at 25×10^{-4} M and rapidly decreasing above 100×10^{-4} M. For a given dehydrogenase system the effects of low concentrations of cysteine and reduced glutathione are of approximately the same order of magnitude suggesting the possibility that it may be the cysteine moiety of the glutathione molecule which is responsible for the latter's activating effect. However, results obtained with various other sulfhydryl-containing substances appear to indicate that the presence of the cysteine moiety in a molecule is not essential in order for it to activate platelet dehydrogenase systems. As is apparent from the data pre-

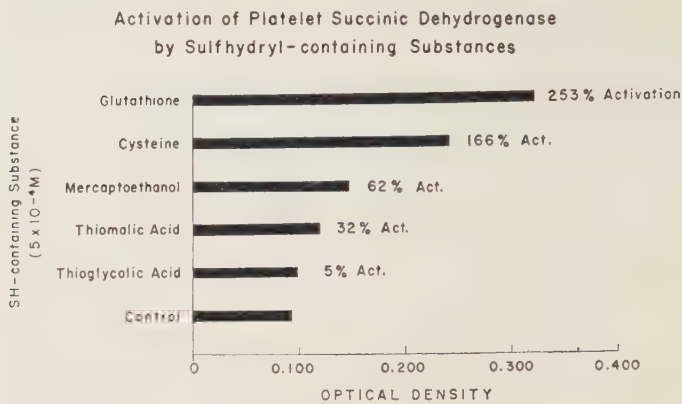


FIG. 3.

sented in Fig. 3 all of the other substances tested exhibit some activating capacity, although none of them even approaches the effect associated with either cysteine or reduced glutathione.

On the other hand, agents capable of oxidizing sulfhydryl groups such as iodosobenzoate and oxidized glutathione as well as mercaptide-forming substances such as p-chloromercuribenzoate and phenylmercuric hydroxide were found to inhibit platelet dehydrogenase activities. However, depending upon their concentration and the length of incubation, these agents also bring about a more or less marked destruction of platelets. The nature of this destruction appears to be different in type from that produced by the previously mentioned lysing agents in that a heavy precipitate is formed leaving an almost transparent supernatant fluid. Whether or not the observed dehydrogenase inhibitions are a direct result of enzyme sulfhydryl inactivation proper or whether they are only a secondary effect due to prior platelet destruction can, therefore, not as yet be stated. What the results do seem to indicate, however, is the existence in the cells of protein sulfhydryl groups, enzymic or not, which are essential for the maintenance of platelet integrity.

Further support for this conclusion is provided by evidence of a different nature. Thus, it has been found that the rate of platelet destruction occurring during storage can be reduced by -SH-compounds or environmental conditions interfering with sulfhydryl oxidation(4). In addition, it has been ob-

served that the yield of platelets obtainable from a sample of versene-collected blood is several times that obtainable from a citrate-collected sample of the same blood and prepared in the same manner(4). Versene may bind metal ions which catalyze the oxidation of -SH groups. Of interest in this connection is a recent report by Benesch *et al.* on the relation between erythrocyte integrity and sulfhydryl groups(8).

Discussion. The finding that lysed platelets are lacking in dehydrogenase activities is surprising since in most other tissue preparations these enzymes are not dependent upon an intact cell structure for their activity. Preliminary work with repeatedly frozen and thawed, densely packed platelets seems to indicate that the observed absence of dehydrogenase activity is due to an inactivation of the enzyme proper. However, not sufficient evidence has been obtained so far to definitely exclude the dilution and disorganization of the complete enzyme system as contributory factors. It is of interest that findings similar to those reported here have been described previously by Penrose and Quastel(9) for a number of dehydrogenase systems in whole and lysozyme-lysed cells of *Micrococcus lysodeikticus*.

The observed activation of platelet dehydrogenase systems by low concentrations of -SH-substances may be explained by assuming that although some enzyme sulfhydryl groups were inactivated (oxidized) during the preparation of the platelet concentrates they could readily be reactivated (reduced) by

cysteine or reduced glutathione. The rapid decrease in activation noted with increasing sulfhydryl concentrations above 100×10^{-4} M is probably due to the gradual build-up of cystine and oxidized glutathione both of which are dehydrogenase inhibitors(10,11).

The fact that platelet dehydrogenase systems can be activated strongly by suitable concentrations of -SH-compounds suggests the possibility that other types of -SH-requiring platelet enzymes may need similar reactivation in order to exhibit maximum activity. The findings that versene improves the stability of platelets and that conditions interfering with sulfhydryl oxidation slow down platelet destruction not only underline the importance of free, active sulfhydryl groups to both platelet integrity and function, but may well explain the discrepancy in the literature concerning the rate of platelet metabolism. It would appear that, in order to prepare platelets with the least possible damage to their structure, inherent biochemical properties and resulting biological activities, conditions of collection and concentration should be employed which minimize damage to essential -SH groups.

Summary. 1. The presence in versene-collected, freshly prepared human platelets of a number of active dehydrogenase systems has been demonstrated. 2. Platelets can be lysed readily by various chemical and physical means, the resulting preparations being homogeneous and opalescent, but completely lacking in dehydrogenase activity. Evidence has been obtained to indicate that platelets have to be morphologically intact for dehydrogenase systems to be active. 3. The dehydro-

genase systems present in platelets are activated by sulfhydryl-containing substances, the most marked effects of those studied being produced by cysteine and reduced glutathione. 4. The addition to platelets of sulfhydryl-oxidizing or mercaptide-forming agents results in platelet destruction apparently different in nature from that brought about by lysing agents not reacting with sulfhydryl groups. 5. Preliminary indications are that platelet destruction occurring during storage can be reduced by sulfhydryl-containing substances or environmental conditions interfering with sulfhydryl oxidation. 6. Possible implications of some of these findings have been briefly discussed.

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Responses of Liver Lipid Fractions, Liver and Plasma Electrolytes Shortly After Alloxan Administration in Rat. (21190)

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The response of the blood sugar to alloxan administration is well documented(1,2). Recently, Nichols and Sheehan(2) clearly demonstrated that the primary and permanent hyperglycemic phases after alloxan are eliminated in dogs with "chemical ablation" of the zona reticularis and fasciculata of the adrenal glands. However, little is known of the nature of the alterations of other chemical constituents of tissues or the factors responsible shortly following alloxan injection which might be relevant to its action on the organism as a whole and the subsequent development of "alloxan diabetes." This paper reports the changes in liver lipids fractions, and liver and plasma electrolytes in groups of rats sacrificed at 4, 24, and 72 hours after intravenous administration of alloxan. Determinations also were made of adrenal ascorbic acid and total cholesterol concentration as indices of adrenocortical activation in these animals.

Materials and methods. Male Wistar rats weighing 250-300 g were used. A 5% solution of Alloxan (Eastman No. 1722) was injected directly into an exposed femoral vein at a dose level of 40 mg/kg body weight in all experimental animals. The control rats received an injection of an equal amount of physiological saline (0.1 ml/100 g body weight) and killed 4 hours later. The rats killed 72 hours after alloxan were fasted approximately 20 hours before the administration of the drug but food and water was available *ad libitum* from then to the time they were sacrificed. The animals of the other groups were not fasted before alloxan; solid food and water were available *ad libitum* until they were sacrificed. The rats were anesthetized with EVIPAL (n-methyl-cyclo-hexenyl-methyl barbituric acid) for all surgical procedures, injections, and the removal of tissues for chemical analyses. The following tissue constituents were determined: Sodium and potassium of liver and plasma with aid of

an internal lithium standard flame photometer; liver glycogen(3,4) as glucose equivalents; plasma glucose(5); adrenal ascorbic acid(6), and adrenal total cholesterol concentration(7). The details of the methods employed at this laboratory for the determination of the liver lipids fractions have been previously reported(8). Total lipids were calculated from weight of residue after distillation of petroleum ether and drying to constant weight; lipid phosphorus by the method of Youngsbury and Youngsbury(9), and Fiske and SubbaRow(10); phospho-lipids derived from lipid-P values $\times 26$; cholesterol fractions by method of Schoenheimer and Sperry (7); and neutral fat content was derived by calculation of difference. Heart blood was obtained by direct puncture in a syringe moistened with one drop of heparin. The blood was centrifuged immediately and the plasma separated off for the chemical determinations. Specimens of liver for glycogen and lipids determination were obtained immediately after the blood sample was secured.

Results. The chemical constitution of liver and plasma secured from rats at 4, 24, and 72 hours after alloxan administration is presented in Table I. Inspection shows that at 4 hours after alloxan there was a significant increase in the liver total lipids content mainly due to an increase in the liver neutral fat fraction, whereas potassium and glycogen content of the liver were significantly decreased. The hyperglycemic condition of these rats was marked and associated with a significantly elevated plasma potassium concentration. Analyses of tissues obtained from the group 24 hours after alloxan revealed that the liver total lipids content was significantly greater than the respective value of the controls. This change in lipids content was due mainly to the marked increase in the liver neutral fat content and to a significant increase in the phospho-lipids fraction. The changes in the liver

TABLE I.

Changes in Chemical Constituents of Tissues following Intravenous Alloxan Administration.

Tissue constituents	Exp. groups—Time after alloxan			
	Controls (10)	4 hr (6)	24 hr (4)	72 hr (6)
Part A. Liver lipids fractions (g %)				
Total lipids	4.96 ± .11	5.32 ± .10*	7.10 ± .57‡	6.27 ± .16 ‡
Phospho-lipids	4.11 ± .08	4.07 ± .09	4.47 ± .07†	4.66 ± .11 ‡
Total cholesterol	.20 ± .004	.21 ± .007	.20 ± .006	.23 ± .009†
Free "	.17 ± .003	.18 ± .001	.17 ± .01	.21 ± .01 ‡
Ester "	.02 ± .003	.03 ± .003	.03 ± .004	.03 ± .005
Neutral fat	.63 ± .09	1.03 ± .09*	2.30 ± .46‡	1.34 ± .26 ‡
Part B. Liver and plasma electrolytes,§ water, glucose				
Liver:				
% water	69.2 ± .13	69.8 ± .2	69.6 ± .6	69.1 ± .12
K	93.5 ± 1.1	88.1 ± 1.5*	81.1 ± 3.9†	82.7 ± 2.0 ‡
Na	23.4 ± 1.0	25.6 ± .5	30.8 ± 3.0*	30.8 ± 1.5 ‡
Glycogen (g %)	3.15 ± .22	1.97 ± .29†	1.54 ± .15‡	1.62 ± .45 ‡
Plasma:				
% water	92.9 ± .09	92.1 ± .17	91.7 ± .2	92.2 ± .2
K	4.60 ± .17	5.60 ± .27†	7.01 ± .32‡	5.30 ± .06 ‡
Na	165.4 ± 3.9	167.7 ± 2.4	153.2 ± 6.3†	155.6 ± 4.3 ‡
Glucose (mg %)	119 ± 4	500†	500‡	500‡

All values are mean ± S.E. Italicized values are significantly different from respective control values. "P" values of significant differences are indicated as follows: * ~.05 to .01; † ~.01 to .001; ‡ ~.001 and less.

§ Liver and plasma electrolytes are expressed as mEq/kg wet tissue and mEq/kg plasma water, respectively.

| Glucose values of experimental groups were at least that shown.

No. of rats in each group are figures in parentheses.

and plasma of the other constituents were similar to that found at 4 hours after alloxan—decreased potassium and glycogen content of liver, hyperglycemia, elevated plasma potassium level, and in addition a significant increased sodium content of liver and decrease in sodium concentration of the plasma.

It is generally agreed that by 72 hours following administration of the drug that an "alloxan diabetes" is established. Reference to Table I shows that the alterations in the chemical constitution of the liver and plasma at this time following alloxan injection were of the same general nature and the changes were comparable to those found in the groups at 4 and 24 hours after administration of the drug. The only additional changes found in the former group and different from the controls and the other 2 alloxan-injected groups were the significant increases in the free and total cholesterol content of the liver. Polyuria and glycosuria were marked in this group, but also were considerably evident in the 24-hour group.

The adrenal ascorbic acid and total chole-

sterol concentrations determined in the control and alloxan-injected groups are presented in Table II. Evidence of depletion of these adrenal chemical constituents is believed to be a reliable index of acute adrenocortical activation. These data therefore indicate a condition of increased adrenocortical activity had been induced in the group killed 4 hours after alloxan injection. The mean ascorbic acid concentration in the group 24 hours after alloxan was not different from the control value but the adrenal cholesterol concentration still was significantly lower in the former group. This type of time-response relationship between adrenal ascorbic acid and cholesterol changes are believed typical of a sudden temporary increased demand for cortical hormones when animals are exposed to a temporary period of stress(11).

Long(12) had expressed the view that increased secretion of the adrenal medulla was a common element in the various forms of stress. Consideration of this proposition and the evidence presented in Table II prompted a study of the chemical constitution of liver

TABLE II. Adrenal Ascorbic Acid and Cholesterol Concentrations in Groups of Rats at Indicated Time-Intervals following Alloxan Administration.

Groups	Right adrenal		Left adrenal	
	Ascorbic acid (mg/100 g adrenal)	"P"*	Total cholesterol (mg/g adrenal)	"P"
Controls (10)	419 \pm 10		35.1 \pm 3.2	
Alloxan inj.				
After 4 hr (6)	259 \pm 8	<.0001	21.4 \pm 3.4	.02
24 (4)	408 \pm 22	ND	14.0 \pm 2.5	.003
72 (6)	397 \pm 27	ND	30.6 \pm 2.3	ND

All values are mean \pm S.E. Figures in parentheses are No. of rats in each group.

* Difference between means occurring by chance compared with controls.

and plasma in adrenal demedullated rats 72 hours after alloxan administration. Adrenal enucleation was accomplished in 20 rats. They were given 1% NaCl solution to drink for the first 10 days and then given tap water for the next 15 days. After an overnight fast 10 rats were injected with alloxan and the remainder with physiological saline. Tissues were secured for chemical analyses 72 hours later. The data are presented in Table III. All the liver lipid fractions, except phospholipids, and the liver and plasma electrolytes were found to be significantly different in the alloxan-injected group from the respective values of the controls. It is notable that the absence of the adrenal medulla and its secretory products did not measurably affect the response of the tissue constituents to alloxan administration. The changes in tissue chemistries were essentially the same in the demedullated and intact groups 72 hours after alloxan.

Discussion. The above results show that at the 3 periods of time chosen to determine the responses of certain tissue constituents to alloxan administration there were certain alterations in the chemistries common to all groups—particularly the increased neutral fat content and decreased potassium content of the liver, and the increased plasma potassium level. The alterations in the liver lipid fractions and the electrolytes of liver and plasma 72 hours after alloxan are those manifestations of the changed metabolism expected with insulin insufficiency. On the other hand, the altered chemistries found in the 4- and 24-hour groups cannot be presumed to reflect solely the change in metabolism in conse-

quence of alloxan's action on the islet beta cells although it is known that alloxan has a toxic action on these cells within 5 minutes of its administration. In regard to these alterations in tissue constitution found in the latter 2 groups the following explanations can be proposed: 1) these changes in tissue chemistries may be manifestations of alloxan poisoning, particularly a hepatotoxic action of alloxan. It is of interest, therefore, that Beach *et al.*(13) expressed the belief that alloxan may damage mechanisms or factors other than insulin as explanation of their results. Recently, Goldner and Jauregui(14) reported evidence from perfusion experiments demonstrating an alloxan effect on the liver. They showed that alloxan inhibits the spontaneous glycogenolysis as well as the adrenalin induced glycogenolysis. The effect upon the liver appeared to be an immediate one. 2) The alterations in liver and plasma chemical constitution reported here might be adrenocortical conditioned "stress" type of responses to alloxan injection at least during the first 24 hours. Indeed, the data presented in Table II show that adrenocortical activation had been induced following alloxan injection. It is evident that further studies are necessary to elucidate the nature of these chemical responses and the factor(s) which may be responsible.

Summary. The responses of the lipid fractions of the liver, and liver and plasma electrolytes to alloxan administration were determined at 4, 24, and 72 hours. The adrenal ascorbic and cholesterol concentrations were also measured as indices of adrenocortical activation in these animals. Besides the known

TABLE III. Response of Tissue Constituents in Adrenal Demedullated Rats 72 Hours after Intravenous Alloxan.

Tissue constituents	Controls (10)	Alloxan inj. (10)	"P" value*
Part A. Liver lipids fractions (g %)			
Total lipids	5.22 ± .11	6.42 ± .22	.0002
Phospho-lipids	4.20 ± .10	4.42 ± .08	ND
Total cholest.	.20 ± .004	.24 ± .01	.002
Free "	.19 ± .003	.21 ± .008	.055
Ester "	.01 ± .004	.03 ± .002	.015
Neutral fat	.75 ± .05	1.74 ± .14	<.0001
Part B. Liver and plasma electrolytes,† water, glucose			
Liver:			
% water	69.3 ± .3	69.6 ± .4	ND
K	86.2 ± 1.0	77.7 ± 1.4	.0002
Na	25.0 ± .8	30.7 ± .9	.0005
Glycogen (g %)	3.60 ± .22	1.56 ± .28	<.0001
Plasma:			
% water	92.8 ± .09	91.9 ± .23	.02
K	5.26 ± .05	5.87 ± .08	<.0001
Na	169.9 ± 2.0	155.6 ± 2.2	.0004
Glucose (mg %)	126 ± 6	500†	<.0001

All values are mean ± S.E. No. of animals in each group is figure in parentheses.

* Difference between means occurring by chance compared with controls.

† Liver and plasma electrolytes are expressed as mEq/kg wet tissue and mEq/kg plasma water, respectively.

‡ All glucose values were at least 500 mg %.

responses of hyperglycemia and decreased liver glycogen content, there were similar changes in other chemical constituents in all groups—an increased neutral fat and decreased liver potassium content, and an increased plasma potassium level. The changes in the tissue chemistries of the 4- and 24-hour groups after alloxan were discussed as signs

of an hepatotoxic action of alloxan, or responses of a stress type which were adrenocortically conditioned. It was also shown that the nature of the altered chemistries 72 hours after alloxan were essentially the same in intact and adrenal-demedullated rats.

The technical assistance of Frank Szematowicz is gratefully acknowledged.

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Virulence of Strains of Herpes Simplex Virus for Mice. (21191)

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It has been our experience that in the use of neutralization tests the HF strain(1) of herpes simplex virus usually yields low and at times inconclusive neutralization indexes (2-4). In spite of the many factors involved, apparently good indexes can be obtained with

some of the recently isolated strains of herpes(2-5). In subsequent reports it will be shown that the strain of virus and the method of preservation are of importance. This paper will report on the virulence of various strains of virus for mice and the ultimate selection of

TABLE I. Strains of Herpes Simplex Virus from Human Sources.

Patient	Age (yr)	Herpetic infection	Reference No.	Virus source	Mouse passages	Incubation 10% suspension (hr)
HF	—	Recurrent	1	Lip	—	30
JM	1	K.V.E.*	2	Skin	9	72 ⁺
FR	16	"	2	"	11	96
HT	23	"	2	"	4	72
EB	25	"	2	"	4	72
CF	1	"	3	"	9	72
RE	2	"	3	"	4	40
GN	22	"	3	"	10	48
AG	8	Rhinitis	4	Blood	9	40
WR	6	Stomatitis	5	Mouth	6	72
PA	5	"	8	"	10	48
BT	57	Recurrent	8	Skin	6	72

* Kaposi's vericelliform eruption (*Eczema herpeticum*).

the RE strain as a suitable test agent. Virulence in mice was tested by the cerebral and peritoneal routes in young and old mice. Moreover, since it has already been shown that inapparent infection may occur after intracerebral inoculation(6), surviving mice in both age groups were tested for immunity in order to see how much of the lower titer in old mice could be attributed to inapparent infection.

Materials and methods. Strains of herpes simplex virus. Strains of virus (excluding HF) were recovered in this laboratory from patients with herpetic infection. The AG strain was isolated by direct intracerebral inoculation of patient's blood into mice. All others were first inoculated onto the scarified corneas of rabbits, and later the infected cornea was transferred to mice by intracerebral inoculation. After several rapid mouse to mouse passages freshly prepared mouse brain suspensions were titrated intracerebrally in mice. Dilutions of virus were prepared in 20% skim milk in saline. The amounts injected were 0.03 cc intracerebrally and 0.2 cc intraperitoneally into groups of 4 or 5 mice. LD₅₀ titers were calculated according to the method of Reed and Muench(7). *Tests for immunity.* At the completion of each titration the surviving mice were tested for immunity to the HF strain of virus. This virus was maintained as a 20% infected mouse brain suspension in undiluted skim milk and stored in dry ice. An ampule was thawed, diluted to 10% with physiological saline and 0.03 cc inoculated intracerebrally. Unused

portions of the ampule were discarded. *Mice.* The mice were albinos bred in our own laboratory. Young animals of either sex weighing between 9 and 11 g were used when 18 to 21 days old. The older mice were at least 6 weeks of age and weighed over 20 g. Although both males and females were used for the older animals only one of the sexes was used for each experiment. Control animals for the immunity tests were approximately the same age, weight, and where possible, the same sex as the test mice.

Results. The histories of the 12 strains of herpes which were used are listed in Table I. Included in the table is the age of each patient, the clinical type of infection and the source material which yielded the virus. The recovered agents were passed several times by intracerebral inoculation of mice until the incubation period had been shortened. The number of these mouse passages required to stabilize the virus is also listed. It was found that the FR strain even after 11 passages still required 4 days to kill mice. The incubation period for 6 of the strains was approximately 72 hours. The PA and GN strains killed the animals in 2 days while the RE required a period of 36 to 40 hours. The laboratory-adapted HF strain required only about 30 to 36 hours to kill mice.

Intraperitoneal titrations in mice. Titrations in intraperitoneally inoculated mice revealed LD₅₀ titers in the old mice which were low; in most cases an end point could not be reached even with a 10% suspension. The results are shown in Table II. On the other

TABLE II. Intraperitoneal Inoculation of Strains of Herpes Simplex Virus into Young and Old Mice.

Strain	LD ₅₀ titer in mice,* 0.2 cc intraperitoneally		Difference, young/old	
	Young†	Old‡	Logs	Fold
HF	3.5	2.0	1.5	32
JM	3.0	.6-	2.4+	>300
FR	3.0	1.0	2.0	100
HT	3.3	2.2	1.1	13
EB	2.7	.7-	2.0+	>100
CF	2.0	.6-	1.4+	> 25
RE	3.5+	1.5	2.0+	>100
GN	3.3	.5-	2.8+	>600
AG	—	—	—	—
WR	4.2+	1.5-	2.7+	>500
PA	3.5	.5-	3.0+	>1000
BT	3.1	.6-	2.5+	>300

* Log of reciprocal of LD₅₀.

† Mice 18-21 days old.

‡ Mice 6 weeks of age or older.

hand, as was expected, the titers in the young mice were higher, ranging between $10^{-2.0}$ and more than $10^{-4.2}$; most of them being in the vicinity of $10^{-3.5}$. The figures in the last column demonstrate the increased susceptibility of the young mice. In a majority of the cases there was a difference of at least 2 logs between the 2 age groups. Animals surviving the intraperitoneal titrations were subsequently tested for immunity by intracerebral inoculation with the HF virus. The results are not recorded since only a few mice survived. This can only be interpreted to mean that intraperitoneal inoculation with living virus was effective in protecting only an insignificant number of mice against cerebral challenge.

Intracerebral titrations in mice. The results of the titrations with the various strains of virus in young and old mice are listed in Table III. Comparisons of results between the 2 groups of animals indicated that the higher LD₅₀ titers were obtained in younger mice. In fact, except for 2 strains, HF and CF, in which the titers were similar, the old mice were consistently more resistant. In 2 instances the differences in resistance were considerable. More than 1000-fold difference was seen with the GN strain and more than 80-fold difference with the PA and RE strains. All of the remaining strains ranged in the vicinity of a 10-fold difference.

The difference in LD₅₀ titers was somewhat narrowed by the subsequent immunity tests.

The survivors of each titration were tested intracerebrally with the HF virus and those found resistant were regarded as having had a previous non-fatal infection. When the number of animals with subclinical infections was added to the number of fatal infections, it was found that, although there was little change in the corrected figures in the young mice, there was a considerable upward revision of LD₅₀ titers (now infective Dose or ID₅₀) in the old mice. It also suggested that the virus generally continued to multiply in the brains of young mice until death occurred, but that in some of the older mice inoculated with comparable dilutions, resistance was sufficient to overcome the infection and render them immune to later cerebral challenge. The differences in intracerebral resistance between young and old mice which previously were marked when mortality was used as a criterion, were now found to be small. Only the BT and RE strains exhibited a difference of one or more logs.

Discussion. Recovery of an infectious agent and demonstration of an antibody rise during convalescence were the means used in this laboratory to establish a diagnosis of herpes simplex infection. Although these objectives were achieved with current procedures, it soon became apparent that there was a great deal of variation among recently isolated strains. Moreover, it also became obvious to us that better neutralization indexes could be obtained with strains of virus other than the HF strain. The RE virus was chosen not only because of its high titer but also because it yielded good neutralization indexes (8). In a later paper it will be shown that various factors come into play to yield good neutralization indexes. Here are presented the results of inoculation into mice of the recently isolated viruses.

Generally, after intraperitoneal inoculation, young mice exhibited higher LD₅₀ titers than old mice. This is in keeping with the observation that resistance to peripheral injection increases with age (9-12). After intracerebral inoculation contrary to expectation young and old mice were not equally susceptible to fatal infection. Higher titers were usually obtained in the young mice and it was some-

TABLE III.
Intracerebral Inoculation of Strains of Herpes Simplex Virus into Young and Old Mice.

Strain	LD ₅₀ titer in mice, 0.03 cc intracerebrally		Fold difference, young/old	ID ₅₀ * intracerebral titers in mice		Fold difference, young/old
	Young	Old		Young	Old	
HF	5.2	5.0	2	5.2	5.2	1
JM	5.4	4.3	13	5.4	4.8	4
FR	4.8	3.8	10	5.0	4.5	3
HT	5.4	4.7	5	5.5	4.8	5
EB	4.5	3.2	20	5.0	4.5+	< 3
CF	5.2+	5.3+	1	5.4+	5.4+	1
RE	6.4+	4.5	> 80	6.4+	4.8	> 40
GN	5.0	2.0-	> 1000	5.0	4.5	3
AG	6.0	5.0	10	—	—	—
WR	5.5	4.5	10	5.7	5.2	3
PA	3.7	1.7-	> 100	4.0	3.5	3
BT	5.0	3.3	50	5.0	4.0	10

* Infective dose₅₀.

what surprising to find that in a majority of instances there were significant differences between the 2 groups of mice. Kilbourne and Horsfall(13) reported on a strain of herpes in which suckling mice were more susceptible than 3-week-old weanlings. However, their titers in suckling mice were no higher than some of the titers in weanling mice reported here. In our experiments an occasional animal developed obvious signs of central nervous system involvement but did not die. This differs from the observation of Andervont (14) that once signs appeared the animal died. Florman and Trader(15) reported titers of 10^{-4} in 25 g mice and undoubtedly use of younger mice would have yielded higher titers.

Summary. Twelve strains of herpes simplex virus, 11 recently isolated, and one, the laboratory HF strain were studied in mice. The incubation periods after intracerebral inoculation of a 10% suspension ranged from 30 to 96 hours. After intraperitoneal inoculation old mice resisted between 20-fold and at least 3000-fold more virus than did the young mice. Comparison of the results of intracerebral inoculation of young and old mice indicated that for 3 of the strains there was no difference; for 9 of them the old mice showed at least a 10-fold greater resistance of which 3 showed a 50-fold or greater difference

and one more than 1000-fold difference. The differences in intracerebral susceptibility to fatal infection were caused by sublethal infection in older mice. When these were taken into account the differences were negligible.

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Biological Demonstration of Estrogenic Substances Excreted in Human Saliva. (21192)

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A biochemical technic for prenatal sex determination was described by Richardson(1, 2). He postulated that salivary glands in women selectively screen out certain female associated hormones but allow male associated hormones to pass into the saliva. If hormones actually are liberated in human saliva and such selectivity does exist, then saliva may represent a new medium for hormone assay in health and disease.

The saliva test for prenatal sex determination has not received adequate clinical verification(3) and no attempt was made in its development to biologically confirm the chemical tests. The presence of hormone-like substances in the saliva has not been established (4).

A survey of the various hormones excreted in saliva in physiological and pathological states has been undertaken. It is our purpose to present biological evidence of salivary excretion of estrogenic substances following intravenous infusion of large quantities of stilbestrol potassium sulfate.

Methods. Two patients were selected for saliva collections, who received, therapeutically, massive doses of stilbestrol, Table I. Saliva specimens were collected following paraffin stimulation, the exact quantity and time of collection noted. These samples were frozen for storage and defrosted prior to hydrolysis. In the first case, the saliva was collected 1) prior to the intravenous infusion of 1000 mg stilbestrol potassium sulfate (given over 9 hr 15 min. period); 2) at the completion of the infusion; 3) 12 hours after completion of infusion. The samples were boiled in 10 cc concentrated HCl for 10 minutes and then extracted with 10 cc of boiling carbon tetrachloride 3 times, the solvent layer having been removed each time and collected. The samples were cooled between each step. Residual water and extraneous material were removed by filtration through anhydrous sodium sulfate. The filtrate was then dissolved

quantitatively in a corn oil vehicle and the carbon tetrachloride evaporated. In the *second case*, the saliva collected after one intravenous infusion of 1000 mg stilbestrol potassium sulfate (given over a 16 hr period) was divided into equal portions. One was treated as described above. The other was allowed to incubate with beta glucuronidase (5000 units/cc), 0.75 cc/30 cc volume, and crystalline penicillin G (10000 u/cc), 0.25 cc/3000 volume for 24 hr at 37°C. The estrogens were then extracted 3 times with 10 cc carbon tetrachloride as described above and similar procedure followed. The exact total volume of the corn oil sample was noted and 0.5 cc of this was given subcutaneously to the test animals on each of 3 days. The technic for estrogen assay was a modification of the method described by Lauson *et al.*(5). Twenty-three-day-old castrated female Holtzman rats were used. A standard curve (Fig. 1) was determined employing crystalline stilbestrol potassium sulfate (Table II) for calculating the estrogenic content of the unknown samples.

Results. Table I indicates that the saliva, collected following the intravenous infusion of 1000 mg of stilbestrol potassium sulfate,

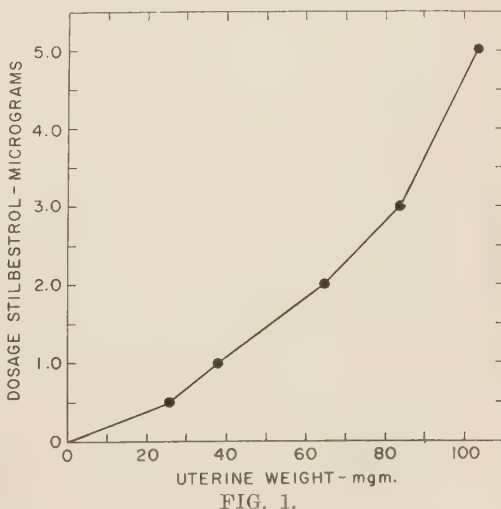


TABLE I.

Patient	Age	Sex	Specimen	Preparation	Treated uterine wt (mg)	Control uterine wt (mg)	Vol of corn oil preparation (cc)	Estrogen present in 1.5 cc of corn oil preparation (μg)	Estrogen in saliva (γ %)
B.G. Prostate carcinoma	69	♂	(1) 101 cc saliva (65 min.) prior to therapy	Acid hydrolysis	16 ± 2		6.0		
			(2) 67 cc saliva (60 min.) following 1000 mg K stilbestrol sulfate IV given 9 hr 15 min.	"	96 ± 6	15 ± 3	6.5	3.8	24.4
			(3) 92 cc saliva (75 min.) obtained after completion of above infusion	"	17 ± 6		6.7		
P.M. Breast carcinoma	76	♀	55 cc saliva (55 min.) (divided in half and prepared by 2 methods) (following 1000 mg potassium stilbestrol sulfate IV given over 16 hr period)	"	29 ± 4	18 ± 1	7.7	.63	5.8
				Glucuronidase hydrolysis	28 ± 4		7.9	.58	5.5

(patient B.G.) contained approximately 24.4 μg % of estrogenic substance. This is in sharp contrast to specimens taken prior to and 12 hours after completion of the infusion. Blood was taken simultaneously with the saliva collection, and contained 55 μg/cc serum.

In patient P.M., approximately 5.8 and 5.5 μg % estrogenic substance was present in the saliva collection following the stilbestrol infusion.

Discussion. It appears that the saliva, so readily collected, might offer a suitable media for the study of other organic and inorganic constituents. Claims of a clinical test for prenatal sex determination, even though theoretical and unverified, do raise the question of whether hormones actually are secreted in the saliva.

This report indicates that following intravenous infusion of large amounts of stilbestrol in either males or females estrogenic substances can be found in the saliva. These limited data warrant no further generalization at this time. Numerous factors, including salivary membrane thresholds for hormone excretion and steroid preparation differences must be investigated before the bioassay of hormones in the saliva assumes any clinical significance.

The correlation of salivary hormone levels with those in the blood and urine may throw additional light on the metabolic pathways of these substances. A study of the qualitative nature of the salivary excreted hormones as compared with those in the urine may reveal differences of diagnostic value. In addition, certain interfering substances found in the urine may be absent in the saliva, thereby eliminating technical difficulties. The saliva of several other patients receiving large amounts of testosterone has been tested bio-

TABLE II.

Dose stilbestrol potassium-sulfate, μg	Uterine wt, mg	Control uterine wt, mg
.5	26 ± 6	
1	38 ± 12	
2	65 ± 16	20 ± 2
3	84 ± 6	
5	104 ± 8	

logically. Preliminary work suggests that androgens may also be excreted in the saliva.

Summary. Estrogenic substance has been biologically demonstrated in the saliva of a male and female patient receiving 1000 mg of stilbestrol potassium sulfate intravenously.

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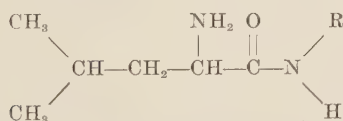
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Specificity of Cathepsin III. (21193)

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The cathepsins(10,11) have been extensively studied by Bergmann, Fruton, and their co-workers(3-5). Several distinct enzymes have been identified by their specific substrate requirements, pH optima, and their different requirements for activation by cysteine or ascorbic acid. This investigation deals with the substrate specificity of cathepsin III (leucine aminopeptidase) of beef kidneys. Several substrates which differ by the size of the group attached to the nitrogen atom of leucine have been utilized to determine the effect of chain length and of polarity upon the activity of the enzyme. The structures of the substrates used are shown by the formula below in which R represents, alternatively, $-H$, $-CH_2COOH$, $-CH_2CH_2COOH$, and $-CH_2CH_2CH_2COOH$.



Methods. Three different enzyme preparations were made by the procedure described by Fruton and Bergmann(3) and were stored at -10°C . Preparation B was activated prior to storage; preparations A and C were activated immediately before use. The enzyme was activated by incubating it for 2 hours at 40°C with 0.01 M cysteine at pH 5. During

activation a heavy flocculent precipitate formed which was removed by centrifugation. The substrate solution was added to the buffered and activated enzyme solution and maintained at 40°C and pH 5 throughout the reaction period. Citrate buffer at a final concentration of 0.04 M was used to keep the pH constant. The initial concentration of substrate in the reaction mixture was 0.05 M. The rate of enzymic hydrolysis was followed by measurement of the liberated carboxyl groups by the micro-titration method of Grassman and Heyde(6). The enzyme concentration is expressed as milligrams of protein nitrogen per milliliter of test solution. The protein nitrogen was determined by a micro-Kjeldahl method described in the Official Methods of Analysis of the A.O.A.C.(8). Non-protein nitrogen was determined on filtrates prepared after precipitation of the protein with 5% trichloroacetic acid. The proteolytic coefficients C_0 and C_1 were calculated from the equation $C = K/E$ where K represents, respectively, the zero order and first order specific reaction rate constants and E is the enzyme concentration. The first order specific rate constant was calculated in decimal logarithms from the formula $K_1 = \frac{1}{t} \log a_0/a$, where t is the reaction time expressed in minutes. The zero order specific rate constant was calculated from the equation $k_0 = \frac{1}{t} (a_0 - a)$.

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TABLE I. Kinetic Data for Hydrolysis of L-leucinamide by Enzyme Preparations A, B and C.

Preparation A *C = .861				B .214				C .552			
t, min.	Hydrolysis, %	$k_1 \times 10^4$	$k_0 \times 10^4$	t, min.	Hydrolysis, %	$k_1 \times 10^4$	$k_0 \times 10^4$	t, min.	Hydrolysis, %	$k_1 \times 10^4$	$k_0 \times 10^4$
60	17.1	13	1.4	60	23.9	19	1.9	30	27.8	47	4.6
120	32.3	14	1.3	120	36.9	16	1.5	60	52.4	53	4.4
180	45.3	14	1.3	180	42.6	13	1.2	90	62.4	47	3.5
240	60.2	19	1.3	240	50.2	12	1.0	120	68.2	41	2.8
300	71.3	18	1.2	300	57.2	12	.9	180	77.6	36	2.2
360	83.1	20	1.1	360	59.9	11	.8	240	78.4	28	1.6

* C = Enzyme concentration (mg protein nitrogen/ml of test solution).

The initial substrate concentration and the substrate concentration at time, t , is represented by a_0 and a , respectively. *Preparation of substrates*—L-leucinamide HCl(9), L-leucylglycine(1), and L-leucyl- β -alanine(7) were prepared by methods described in the literature. L-leucyl- γ -aminobutyric acid was prepared by the same method described by Smith(7) for the preparation of L-leucyl- β -alanine. The crystals of L-leucyl- γ -aminobutyric acid melted at 164°-165°C.

Results. The data for the hydrolysis of L-leucinamide by each of the enzyme preparations are shown in Table I. During the first 45% of its course the hydrolysis of this substrate by enzyme preparation A appears to follow either zero order or first order kinetics; during the remainder of the reaction period it conforms more closely to zero order kinetics. The hydrolysis of L-leucinamide by either preparation B or preparation C follows neither first order nor zero order kinetics; nor does it adhere to the mixed first and zero order equation obtained by Elkins-Kaufman and Neurath(2). Calculation of the proteolytic coefficient from either the average zero order or the average first order reaction rate constants shows preparations B and C to be about 4 times as active as preparation A. The rapid decrease in the rate of hydrolysis of these 2 preparations may be caused by destruction of the enzyme in the more highly purified preparation.

With each of the enzyme preparations the kinetics of the hydrolysis of L-leucylglycine, L-leucyl- β -alanine, and L-leucyl- γ -aminobutyric acid were similar to the kinetic order found for L-leucinamide. The reaction is apparently more complex than a simple first or

zero order kinetic. More exact studies require a more highly purified and more stable enzyme preparation.

Table II summarizes the results for the hydrolysis of the substrates by each of the enzyme preparations. In each instance L-leucinamide is hydrolyzed 2 to 3 times as fast as either L-leucylglycine or L-leucyl- γ -aminobutyric acid and about 4 times as fast as L-leucyl- β -alanine. The slower rate of hydrolysis of L-leucyl- β -alanine cannot be explained by a possible inhibitory effect of liberated β -alanine, since the addition of β -alanine to a test solution in which L-leucinamide was the substrate had no effect on the rate of hydrolysis of L-leucinamide.

Discussion. Since the rates of hydrolysis of L-leucylglycine, of L-leucyl- β -alanine and of L-leucyl- γ -aminobutyric acid were approximately the same, especially with enzyme preparations B and C, the decrease of the polarity of the substrate produced by the greater distance between the carboxyl group and the peptide bond has little effect on the rate of hydrolysis. The faster hydrolysis of L-leucinamide may be the result of a faster rate of enzyme-substrate complex formation permitted by the absence of relatively large groups adjacent to the amide group. In other words, L-leucinamide would be expected to have much less steric hindrance than any of the other substrates.

Summary. 1. A purified cathepsin III (leucine aminopeptidase) preparation hydrolyzed L-leucinamide from 2 to 3 times as fast as either L-leucylglycine or L-leucyl- γ -aminobutyric acid and about 4 times as fast as L-leucyl- β -alanine. The activity of the enzyme was not affected by the proximity to the pep-

TABLE II. Comparative Activity of Cathepsin III toward L-leucinamide and L-leucylpeptides.

Substrate	Preparation A			B			C		
	Enzyme conc.	$C_1 \times 10^4$	$C_0 \times 10^4$	Enzyme conc.	$C_1 \times 10^4$	$C_0 \times 10^4$	Enzyme conc.	$C_1 \times 10^4$	$C_0 \times 10^4$
L-leucinamide	.861	20	1.4	.202	76	7.3	.552	76	5.8
L-leucylglycine	.805	6.4	.60	.205	18	1.9	.565	18	1.8
L-leucyl- β -alanine	.852	3.9	.43	.205	13	1.4	.562	13	1.4
L-leucyl- γ -amino-butyric acid	.861	6.4	.63	.200	21	2.2	.567	18	1.8

Enzyme conc. = mg protein nitrogen/ml of test solution.

C_1 = First order specific reaction rate constant/enzyme conc.

C_0 = Zero

tide bond of the free carboxyl group of leucyl peptides. 2. The fact that L-leucinamide is hydrolyzed faster than are the peptides of leucine can possibly be explained by the hypothesis that the large groups attached to the peptide nitrogen of the substrates interfere with the formation of an enzyme-substrate complex. 3. The hydrolysis of the substrates by enzyme preparation A closely approximated zero order kinetics. The failure to observe a regular kinetic order for the reactions catalyzed by preparations B and C may be the result of destruction of the enzyme in the more highly purified preparations.

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Reversal of Cortisone Inhibition of Wound Healing by Tissue Culture Media.* (21194)

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The inhibition of normal wound healing by cortisone was demonstrated by Howes(1) and Spain(2). As early as 1911, Carrel demonstrated growth promoting and sustaining factors in embryo extracts as applied to tissue cultures of mammalian cells(3). The purpose of this paper is to present data on the influence

of media containing Carrel's growth factors on cortisone inhibition of wound healing.

Methods. Under light ether anesthesia wounds 3 cm in length were incised through the skin to the fascia of the back of the neck of young adult male and female rats of the Sprague-Dawley strain. In one group single wounds were made. In a second group 2 parallel incisions 2 cm apart were made. The wounds were studied under 3 different conditions, as follows: 1) Wounds which were

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FIG. 1. Normal wound 8 days, $13\frac{1}{2} \times$, Mallory stain.

painted 10 times or injected twice daily with Hank's balanced salt solution were considered "normal wounds"; 2) Wounds produced on rats treated daily with a single subcutaneous injection of 10 mg of cortone acetate (Merck & Co.) were considered as "cortisone inhibited wounds"; 3) Wounds which were painted 10 times daily or injected twice daily with tissue culture media while the animals received the same dose of cortone acetate as depicted in 2) were considered as wounds showing reversal of cortisone inhibition of wound healing. Some of the wounds of groups 2) and 3) were inflicted in the same animal. The tissue culture media consisted of either defatted embryo extract prepared as a 50% solution in Hank's balanced salt solution or

beef amniotic fluid obtained from a freshly slaughtered cow which was 2 months pregnant. The experiments extended over 6 to 14 days.

Results. Fig. 1 depicts a normally healing wound at 8 days. It shows normal granulation tissue formation permeated by moderate numbers of chronic inflammatory cells. The surface of the wound is covered by a scab of fibrin, dead leucocytes and red cells. All of the 14 normal wounds were similar in appearance.

Fig. 2 depicts a cortisone inhibited wound at 8 days. It shows virtually no granulation tissue or chronic inflammation. The scab appears the same as in Fig. 1. There were 35 cortisone inhibited wounds; all had the same appearance.

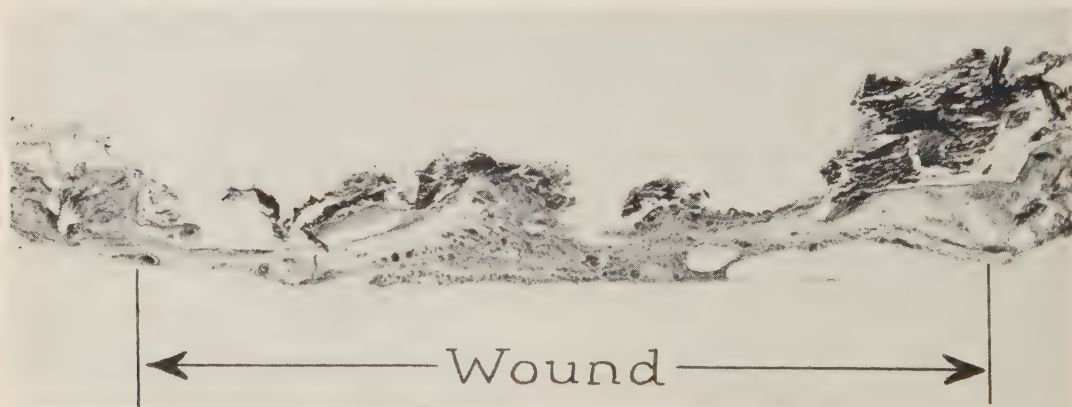


FIG. 2. Cortisone inhibited wound, 8 days, $13\frac{1}{2} \times$, Mallory stain.

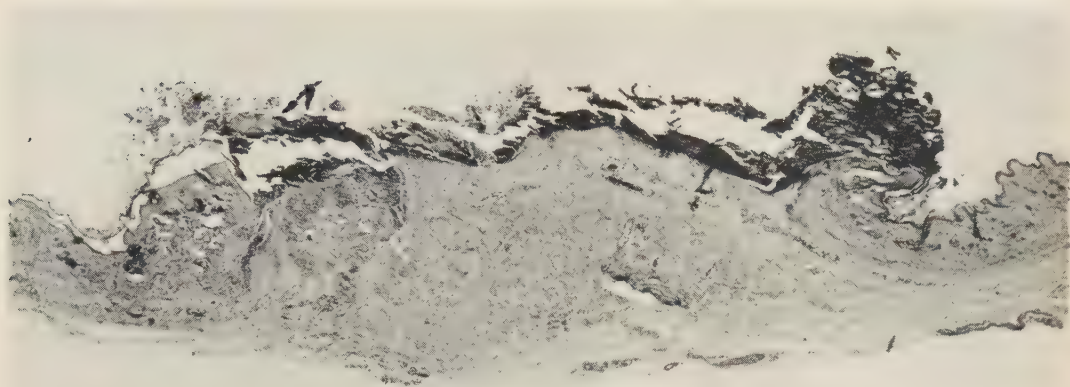


FIG. 3. Reversal of cortisone inhibition, 8 days, $13\frac{1}{2}\times$, Mallory stain.

Fig. 3 depicts the reversal of cortisone inhibition of wound healing by tissue culture media at 8 days. It shows abundant granulation tissue with a more pronounced deposition of collagen than present in the normal wound. The 32 treated wounds were of similar appearance.

Fig. 4 depicts a gross photograph illustrating systemic cortisone inhibition of wound healing on the left and reversal of cortisone inhibition on the right. Both wounds were 14 days old.



FIG. 4. Systemic cortisone inhibition—left. Reversal of cortisone inhibition—right. Both wounds 14 days old.

Three wounds show some escape from cortisone inhibition of wound healing. This is manifested by a moderate amount of granulation tissue and some chronic inflammation. These wounds are not comparable in their degree of healing to the normal nor the wounds treated with tissue culture media. Three wounds treated with tissue culture media fail to show complete reversal of cortisone inhibition of wound healing. These wounds resembled the wounds showing some escape from cortisone inhibition. The results are summarized in Table I.

The granulation tissues of these 3 types of wounds contain the following substances as demonstrated by a positive histochemical reaction: neutral fats, fatty acids, tri-glycerides, phospholipids, phosphatides, tyrosine, alkaline phosphatase, iron(4), non-specific esterase (5), potassium, free aldehyde groups, glycogen, calcium(4), free carbonyl groups(6), and protein bound sulphydryl groups(7). Mast cells were present in equal numbers in all sections. These tests were less positive in the cortisone inhibited wounds because these wounds contain little or no granulation tissue.

Discussion. Other workers have indicated that the active growth promoting principle of embryo extract may be a pentosenucleoprotein (8-11). It is conceivable that the active component in the crude extracts used in these experiments is of a similar nature. If so these experiments lend some support to Carrel's hypothesis that chronic inflammatory cells liberate growth promoting substances which

TABLE I. Results of Wounds in Groups 1, 2 and 3.

Normal	14
Cortisone inhibited	35
Reversal of cortisone inhibition	32
Failure of complete cortisone inhibition	3
Failure of complete reversal of cortisone inhibition	3
Total	87

induce wound healing(12). Cortisone would then appear to function as an inhibitor of wound healing by virtue of its ability to modify inflammation(1,2).

Summary. 1. Local applications of tissue culture extracts will completely reverse cortisone inhibition of wound healing. 2. The granulation tissues of these wounds are histochemically similar.

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Adrenocortical Function in Hypo- and Hyperthyroidism.* (21195)

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Adrenal cortical hypertrophy following thyroid administration has been demonstrated many times since the work of Hoskins on the guinea pig(1). Its dependence on the presence of the pituitary was shown by Smith, Greenwood and Foster(2) in the course of metabolic studies with small doses of thyroxine in rats. Conversely, the cortical atrophy following thyroidectomy has frequently been noted (*e.g.* Evans, Simpson, and Pencharz (3)). The morphological picture of cortical atrophy resulting from thyroidectomy, and hypertrophy from treatment with thyroid hormone in the rat, has recently been illustrated by histological and cytochemical methods by Deane and Greep(4) and by Feldman(5). The question of the functional response of the adrenal cortex in hypothyroidism has been raised by Hill *et al.*(6) on the basis of impaired eosinopenic response to the ACTH test (Thorn *et al.*(7)) in certain hypothyroid patients and its improvement following thyroid

hormone therapy in some of these patients. The ACTH test was adapted to use in experimental animals (dogs and rats) by Recant *et al.*(8). It offered a promising approach to the study of adrenocortical function in thyroid deficient and hyperthyroid rats. The work of Gabrilove and Soffer(9) on the ascorbic acid depletion response of propylthiouracil treated rats suggested another approach.

The plan followed in the present work was to study: A) the eosinopenic response to various stimuli such as ACTH or epinephrine, and B) the ascorbic acid depletion response to ACTH, in hypothyroid rats before and after thyroxine therapy, as compared with normal rats, and with normals rendered hyperthyroid by thyroxine administration.

Procedure. Male rats of the Long-Evans strain were surgically thyroidectomized at 21 days of age. After a prolonged interval, those which had shown a marked retardation in growth, and various external signs of thyroid deficiency (such as thinning of hair and accumulation of subcutaneous fat) were further screened for completeness of thyroidectomy

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by determination of metabolic rate. (This was done by means of a simple apparatus for measuring oxygen consumption in terms of the volume of water replacing it dropwise in a closed system. Basal conditions were approximated as closely as possible by training the animals in the apparatus, and by the use of a 20 to 24-hour fasting period before the determination.) Control standards for metabolic rate were developed on groups of intact males of the same age. These had an average fasting body weight of 450 g and a metabolic rate of 41.4 ± 1.57 Cal/m²/hr. The thyroidectomized rats selected for use in the experiments to follow averaged 196 g in body weight and had metabolic rates averaging 27.5 ± 0.69 Cal/m²/hr or 36% below normal. The average rate was raised approximately to the normal value (41.1 ± 1.34 Cal/m²/hr) in 14 days, by a daily dose of 0.005 mg thyroxine (Squibb). A group of normal rats was given a daily dose of 0.100 mg thyroxine for 10 days, resulting in an average metabolic rate of 51.3 ± 1.58 Cal/m²/hr, or 19% above control values, with definite signs of hyperthyroidism. *Eosinophils* were counted directly by the method of Dunger(10), as modified by Recant *et al.*(8). Materials used to evoke the eosinopenic response were ACTH and epinephrine. The ACTH preparation used (Armour, Lot 71-2 (50) c) was potent in the adrenal weight maintenance test at a daily dose of 1.0 mg for 15 days, in hypophysectomized 40-day-old male rats. The minimum dose for adrenal ascorbic acid depletion was 0.5 μ g intravenously. The epinephrine used was Adrenaline chloride (Parke, Davis and Co.) 1:1000 in aqueous solution; a fresh ampoule was opened for each experiment. The *adrenal ascorbic acid* depletion tests were carried out after the method of Sayers *et al.*(11). The hypothyroid rats used were some of those previously used in eosinophil tests, at which time they had received thyroxine therapy. A sufficient interval had elapsed before the ascorbic acid test, so that all the effects of therapy had been lost, and the rats had regressed to the hypothyroid state. As a terminal check on completeness of thyroidectomy, the radioactive iodine uptake of the neck tissues was measured in a scintillation counter.

Most of the thyroidectomized rats had iodine uptakes less than 1% of the normal uptake. Six of the 19 reported had uptakes 5 to 9% of normal, but responded in no way differently from the remainder of the group, justifying their inclusion as thyroid deficient.

Results. A. The Eosinophil test. Before the effects of ACTH or of epinephrine could be measured, it was found necessary to minimize the effect of handling and withdrawal of blood from the tail tip (an effect amounting to a 30 to 50% decrease in circulating eosinophils). This was accomplished by use of a mild sedation with nembutal (Abbot, veterinary) given initially at a dose of 3 mg/100 g body weight, intraperitoneally, 2 hours before the taking of the first blood sample. Stabilization was dependent upon the *renewal* of sedation by small supplementary doses at hourly or half-hourly intervals, as required (after the method of Recant *et al.*(8), as described by P. H. Forsham in a personal communication). Otherwise, the administration of a single dose of nembutal lowered the eosinophil count, as reported by a number of workers (Sawyer and Parkerson(12); Dumm and Ralli(13)). The eosinophil count was stabilized in this way, so that not more than 5 to 15% of the decrease was attributable to the sampling technique, in all rats being tested with ACTH or epinephrine.

The results of intraperitoneal injection of 3 mg of ACTH are shown in Fig. 1. The

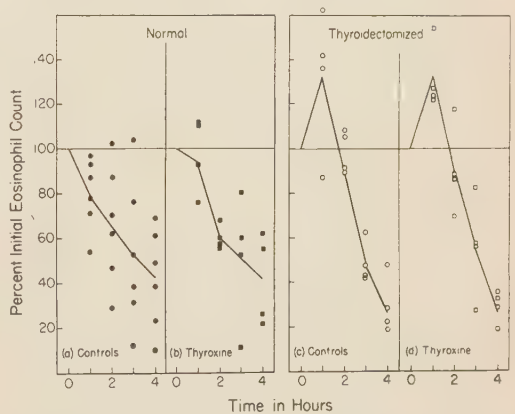


FIG. 1. Effect of ACTH on circulating eosinophils of normal and thyroidectomized male rats, with and without thyroxine treatment. (Nembutal sedation.)

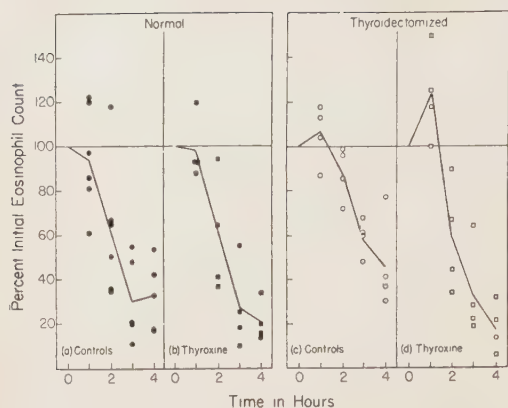


FIG. 2. Effect of epinephrine on circulating eosinophils of normal and thyroidectomized male rats, with and without thyroxine treatment. (Nembutal sedation.)

eosinophil counts of the normal rats showed an average fall of 58% (± 8.02) in 4 hours, and a comparable fall, 62% (± 8.9) was obtained in a normal group receiving thyroxine. The thyroidectomized rats responded with a decrease of 73% (± 4.18) before thyroxine therapy and 72% (± 3.48) afterwards. There was, then, no impairment of the ability of the adrenal cortex of the thyroidectomized rats to respond to exogenous ACTH, as measured by this test.

When 0.1 mg epinephrine was used as the stimulus, normal rats responded with a decrease of 73% (± 6.22) in eosinophil count (Fig. 2). This response was unaffected by thyroxine treatment ($82\% \pm 3.18$). The thyroidectomized rats showed some impairment in this response, the average decrease being 56% (± 8.58). Thyroxine therapy had a definitely beneficial effect on the response to epinephrine, resulting in an average decrease of 85% (± 3.34). This improvement was even more clearly seen when the responses of individual rats, before and after thyroxine treatment, were compared as in Fig. 3. The difference between the responses of thyroidectomized and normal rats to epinephrine was not statistically significant, due to the great individual variation. However, the difference between the thyroxine-treated and untreated thyroidectomized rats was highly significant, and the response of the thyroxine-treated group was equivalent to normal.

B. Ascorbic acid depletion test. For the

adrenal ascorbic acid depletion test, a dose of 5 μ g of the adrenocorticotrophic preparation was chosen, as this was in the significant region of the standardization curve. The assay was carried out in the routine manner, 24 hours after hypophysectomy in 7 "standard" animals (40 days old), in 11 of the thyroidectomized rats and 6 normal controls of the same age, and in 3 hyperthyroid rats.

Table I shows that the decrease in ascorbic acid in the thyroidectomized rats was greater and more uniform than in the normal rats of the same age (132 ± 5.41 mg/100 g adrenal as compared to 83 ± 14.1 mg). Values for the 3 hyperthyroid rats which survived the procedure fell within the normal range. It may also be seen in Table I that the response of the thyroidectomized rats was greater, and that of their normal age controls slightly less, than that of the younger "standard animal".

Discussion. So far as the eosinophil test was concerned, the thyroidectomized rats showed no impairment of adrenocortical response to exogenous ACTH. Impairment of the response to epinephrine would seem to place the site of weakness in the pituitary-adrenocortical system at the pituitary rather than the adrenal level. Evidence for lessened pituitary content of ACTH in thyroidectomized rats has been presented by Halmi and Bogdanove (14). Specificity of the eosinopenic response to epinephrine as an indicator of adrenocortical function has been questioned (15), and thus the significance of the impaired

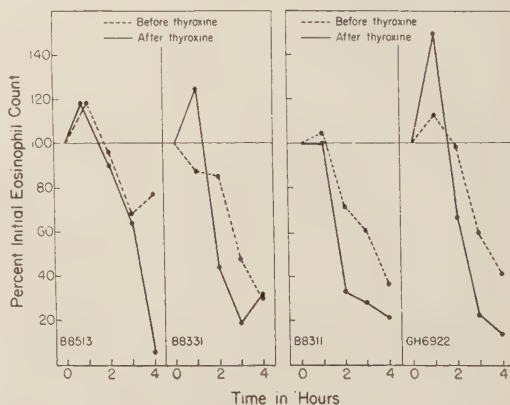


FIG. 3. Individual responses of circulating eosinophils of thyroidectomized male rats to epinephrine, before and after thyroxine treatment. (Nembutal sedation.)

TABLE I. Effect of Thyroid Status on Adrenal Ascorbic Acid Depletion Response to a Standard Dose of ACTH (5 μ g per 100 g BW, Injected IV).

Type of rat*	No. of rats	BMR, Cal/m ² /hr	Body wt, g	Adrenal wt, mg		Adrenal ascorbic acid, mg/100 g adrenal		Depletion
				Left	Right	Left	Right	
Thyroidectomized†	11	26.6	279	13.5	13.5	276	146	132 \pm 5.41
Normal controls†	6	37.7	439	22.3	20.3	298	215	83 \pm 14.1
Normal + thyroxine†	3	53.7	454	23.4	20.9	309	224	86 \pm 15.2
Normal 40-day	7	—	119	10.6	10.3	448	348	100 \pm 13.7

* All rats hypophysectomized 24 hr before the test.

† Approximately one year old at time of this test.

response to epinephrine, and its improvement by thyroxine, is not entirely clear.

The adrenal ascorbic acid depletion test indicated an enhanced adrenocortical responsiveness in the hypothyroid state. Finerty and Hess(16) have found an increased ascorbic acid response to the stress of scalding, in thyroidectomized rats. However, caution should be used in the interpretation of all such results, since, as has been pointed out by Freedman and Gordon(17), the same or a slightly greater response *per unit weight of gland* (as the ascorbic acid depletion is reported) may still mean a greatly reduced daily hormonal output from a small as compared with a large adrenal.

Summary. 1. As a means of assessing adrenocortical function, the eosinopenic and adrenal ascorbic acid depletion responses of normal and thyroidectomized male rats, with and without thyroxine treatment, have been examined. 2. With nembutal sedation, the eosinopenic response to ACTH was the same in thyroidectomized as in normal rats and was not influenced by treatment with thyroxine in either group. 3. Under these conditions the eosinopenic response to epinephrine was less in thyroidectomized than in normal rats and was distinctly improved after thyroxine therapy. The response of normal rats to epinephrine was not modified by the administration of thyroxine in doses leading to hyperthyroidism. 4. In the second test used, adrenal ascorbic acid depletion in response to ACTH, the thyroidectomized rats showed a greater and more uniform depletion than did normal rats, with the same dose of ACTH. Hyperthyroid rats responded like the normals.

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Dose Dependence and Sequential Changes in Mouse Small Intestinal Weight Induced by Ionizing Radiation.* (21196)

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The marked sensitivity of the gastrointestinal tract and particularly the small intestine of the rat to ionizing radiation has been recently demonstrated quantitatively by measuring weight changes in components of the gastrointestinal tract at various times after irradiation(1). Further work has shown that the mouse small intestine responds in a similar manner (though to a less extent) and that the degree of weight loss, when measured 2 days postirradiation, was dose dependent throughout the lethal range. This technic was designed and used successfully for biological measurement of rem dosage of neutron radiation in atomic bomb field tests. Studies of this dose dependent response of the small intestine of mice to 2000 KVP X-radiation and gamma radiation (Co^{60}) and the sequential weight changes with the latter radiation are presented in this paper.

Materials and methods. Male mice weighing between 18 and 28 g, of the CF (Carworth Farms) strain, were used in the study with 2000 KVP X-ray and male white Swiss strain were used in the gamma-ray study. All animals were randomly selected into control and experimental groups. *Groups of CF mice* were exposed, each mouse in a single cell, in a masonite-wooden constructed unit which was curved to conform to an arc of a circle at 2 meters distance from the radial beam of a 2000 KVP G. E. Industrial X-ray machine (see previous publication(2) for details of this exposure unit). Groups of 17 mice were exposed to doses of 400, 600, 800, 950, 1100, 1300, and 3000 r. Thirty-four control mice were placed in the exposure cages for comparable lengths of time but not irradiated. Radiation factors were: 2000 KVP, 1.5 ma; no added filter; intensity of the radial beam was $15.0 (\pm 0.15)$ r per minute in air at 2

meters; h.v.l. 4.3 mm Pb. The mean effective energy of the beam was .58 Mev. The mice in this study were all sacrificed at 2 days postirradiation for gastrointestinal weights. Preliminary work showed that the 2-day time period gave the best index of dose dependence in gut weight. *Groups of white Swiss mice* were exposed to gamma-ray doses of 150, 350, 550, 750, and 950 r for each of the following sequential time studies: 12, 24, 36 hrs, and 2, 3, 4, 5, 7, 10, and 12 days postirradiation. Five experimental and 5 control animals were used for each group, except for the larger dose and time periods where extra experimental mice were used. This study was repeated about a month later in parallel using the same number of mice. The gamma-ray dose was delivered in a constant time of 10 minutes from an approximately 4π source of cobalt⁶⁰ (1.23 Mev) irradiator(3). The 30-day LD_{50} for these mice with this radiation was 690 r. Doses were measured in air with cages in place by a Victoreen roentgen rate meter using a 4 mm lucite cap. The cobalt was housed in aluminum capsules which filtered out the incident beta radiation. The radiation volume was uniform throughout an area 6 inches in length by 9 inches in diameter. The various prescribed doses were obtained by varying the number of cobalt capsules. In both studies the mice were sacrificed with chloroform and the small intestine was stripped free of mesentery after severing at the pyloric sphincter and ileocecal junctions respectively. The intestines were then opened, washed, and placed in small tared aluminum pans and placed in a 95°C oven and weighed after 18 hours. This time period proved adequate to achieve constant dry weights. The mean dry weights of each group were expressed as the percentage of the mean dry weight of the unirradiated controls. In the gamma-ray study, data from the 2 parallel studies were combined, since an analysis of variance showed that this was statistically valid. (Since this study acted as a

* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

TABLE I. Effect of Radiation on Mouse Small Intestinal Weight. Mean intestinal wt (mg) \pm stand. error of mean.

Time after irrad.	No. mice /group*	2000 KVP							
		0 r	400 r	600 r	800 r	950 r	1100 r	1300 r	3000 r
Controls	34	313±6.9							
2 days	17		258±6.2	234±5.9	215±7.6	193±6.1	194±7.3	188±6.2	183±5.3
		Gamma radiation							
		0 r	150 r	350 r	550 r	750 r	950 r		
Controls	99	263 ± 8.3							
12 hr	10		261 ± 7.7	252 ± 5.7	259 ±11.4	257 ± 9.0	231 ± 9.3		
24	10		261 ±12.6	247 ± 8.9	238 ±11.5	227 ± 9.1	241 ± 8.8		
36	10		257 ± 6.6	252 ±11.5	227 ± 7.5	212 ± 7.1	214 ± 8.7		
2 days	10		261 ±10.6	238 ± 7.9	235 ± 6.5	200 ± 8.0	174 ± 8.5		
3	10		247 ± 6.2	264 ±11.2	277 ±12.3	228 ±19.5	170 ± 9.6		
							(9 of 10)		
4	10		264 ±16.3	256 ±13.3	290 ±10.9	296 ± 5.9	237 ±15.7		
							(7 of 10)		
5	10		294 ±16.6	285 ± 9.6	249 ± 5.4	302 ±11.0	293 ±15.6		
							(8 of 10)		
7	10		269 ± 9.2	266 ±11.9	272 ±11.5	267 ±23.4	266 ± 8.6		
				(9 of 10)		(9 of 10)	(17 of 18)		
10	10		259 ± 9.9	272 ±12.1	266 ±11.5	234 ±10.3	198 ±16.4		
							(5 of 15)		
12	10		265 ±13.2	293 ± 7.9	266 ±18.9	237 ±19.6	223		
						(9 of 10)	(1 of 20)		

* Where numbers of mice in a group differ due to deaths or added mice, number of living followed by original number is presented in parenthesis after stand. error of mean.

control for another study, the mice received isotonic saline (1 ml/100 g wt) subcutaneously 25 minutes before irradiation).

Results and discussion. Table I shows the mean small intestinal weight and the standard error of the mean of groups of mice at various doses and times after 2000 KVP X-ray and gamma radiation. These data indicate a relation between the 2-day small intestinal weight and the amount of radiation that is described by the equation: $Y = a - bx$, where Y is intestinal weight and x equals dose in r. Using the variation in individual gut weights within groups as the error variance, this equation was found to adequately represent the relation between small intestinal weight and the dose of radiation. In Fig. 1, this relation has been converted into percentage values. Both studies showed dose dependence of intestinal weight loss through 950 r. Least square lines have been fitted to the data through 950 r.

The difference in response of the Swiss gamma-treated and the C. F. 2000 KVP X-ray treated mice may be due to strain differences. However, the difference may be partly due to

difference in tissue dose from the two types of radiation.

In the 2000 KVP X-ray experiment, it can be seen (Fig. 1) that at 950 r a maximum weight loss of the intestine was attained on the second postirradiation day and a plateau effect developed with higher doses. At about this dose and above, one also sees early deaths (4 to 6 days postirradiation) in mice which is believed to be associated with severe gastrointestinal damage(4-6). Thus, a radiation dose of around 950 r and above probably represents a critical level of gastrointestinal damage which invokes the early mechanism of death. Williams'(7) studies on mitotic inhibition in the mouse small intestinal epithelium after X-irradiation indicate that doses of this magnitude also produce a maximum suppression of mitosis at about the same time (2 days). Comparing 2-day small intestinal weight loss in sexually mature Sprague-Dawley rats using 200 KVP X-ray with such changes in mature C. F. mice using 250 KVP X-ray, the rat intestine turns out to be about 1.7 times as sensitive as the mouse intestine based

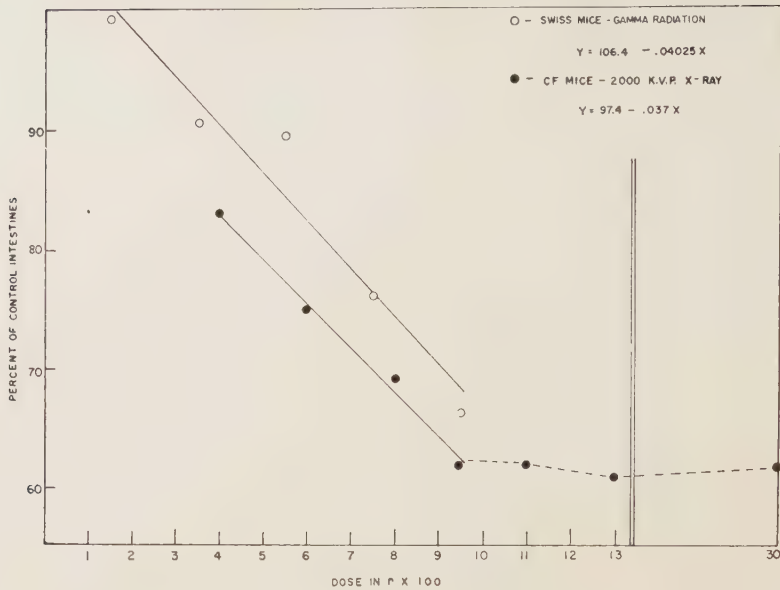


FIG. 1. Effect of irradiation on mouse small intestinal weights 2 days post-irradiation.

on this criterion. This conforms to the gross and histological observations that rats have more sensitive gastrointestinal tracts and exhibit early deaths at lower percentage mortality doses.

Radiation-induced weight loss in the thymus and spleen has also been shown by Carter, *et al.* (8) to be dose dependent when measured 5 days postirradiation and both have been used extensively as biological dosimeters. Body weight loss has also been shown to be an index of radiation dosage (9,10). This is probably a more complicated resultant of radiation damage, whereas weight loss in the thymus and spleen represents primarily lymphoid degeneration and in the gut is associated primarily with epithelial damage.

Sequential weight changes of groups of mice after exposure to various doses of gamma radiation are shown in Table I and Fig. 2. Since the 150 and 350 r doses showed the characteristic responses to a considerably less degree, only the curves for 550, 750, and 950 r were plotted. It can be seen that the gut lost weight rather rapidly to the second and third day, the maximum weight loss being somewhat later with increasing dose. This initial weight loss increased with dose to 950 r and as was shown above, the second day

weight loss serves as a useful index of radiation dose. It has been pointed out (1) that this initial weight loss was believed to be largely due to destruction as well as failure of normal replacement of epithelium due to mitotic inhibition.

The initial weight loss was followed by recovery of weight with an overshoot above the control range, in all doses used. Recovery began somewhat later with increasing dose, the recovery peak tending to be delayed. The degree of overshoot appeared to increase with dose and initial weight loss up to 750 r. Thus, up to 750 r, it would appear that the amount of overshoot is in some way related to the amount of weight loss induced. However, even with 950 r (in the mice that did not succumb to early death) there was remarkable ability of the intestine to regenerate as shown by the 5-day recovery above the control range. Again, in regard to mitotic studies referred to above (7), it is interesting that there appeared to be an increase in mitosis above the normal rate in the gut epithelium which corresponded in general with the dose and time involved in these weight studies.

By the seventh day following the recovery peak, the intestine returned to the control weight range with all doses and remained with-

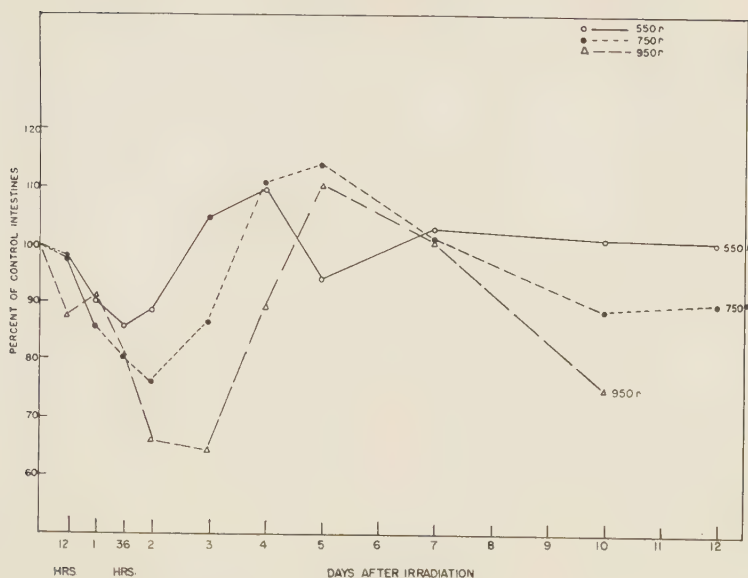


FIG. 2. Sequential weight changes of small intestine of mice exposed to various doses of gamma radiation.

in this range with doses through 550 r. However, with the 750 r and 950 r doses, a secondary depression of gut weight occurred, on the 10th and 12th days. However, (Table I) only survivors can be included with the higher doses (less than 10 animals) at later times of sacrifice, which would if anything lead to an underestimation of the weight loss at this time. This secondary weight loss may be related to general nutritional changes associated with the sequelae of pancytopenia which is believed to be responsible for mortality at this time.

The technic of measurement of radiation-induced weight change in the small intestine of animals, if used under properly controlled conditions, is a useful tool in the field of biological dosimetry. One may determine dosage or rem, relative biological effectiveness of various types of radiation, strain and species differences in response, effects of protective agents, and the relative sensitivity of the gastrointestinal tract to various qualities of radiation compared to other tissues.

Summary. The effect of 2000 KVP X-ray and gamma (Co^{60}) irradiation on the weights of the small intestine of C. F. and white Swiss mice was presented. Weight loss in the small

intestine showed dose dependence when measured 2 days postirradiation with doses of 150-950 r. Doses above 950 r showed no further weight loss at 2 days resulting in a plateau effect. It was pointed out that under properly controlled conditions, this technic may be used as a biological dosimeter in determining dosage or rem, relative biological effectiveness of various types of radiation, strain and species differences in response, effects of protective agents, and the relative sensitivity of the gastrointestinal tract compared to other tissues. Sequential weight changes in the small intestine were studied after various times and doses of gamma radiation in white Swiss mice. Radiation was followed by rapid weight loss in the gut (dose dependent) reaching a maximum by the 2nd or 3rd day followed by recovery above the control range at 3 to 5 days. The magnitude of this peak recovery tended to increase with dose up to 750 r and to be somewhat delayed with increasing dose. These findings showed correlation with similar time and dose studies on mitotic inhibition of the mouse small bowel epithelium(7). By the 7th day the intestines returned to the control range and remained in the range with doses through 550 r. However with doses above 550 r a secondary depression in gut weight

occurred which was manifest on the 10th and 12th postirradiation days.

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Penetration and Local Effect of Vitamin A on the Skin of the Guinea Pig.*†

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Vitamin A penetrates the intact skin of the guinea pig largely by way of the pilosebaceous canals and the sebaceous glands, and the speed of its penetration is dependent upon the vehicle used. Topical applications of massive doses of vit. A over a period of 2 weeks cause an hypertrophy of the epidermis but no appreciable inhibition of keratinization. The growth of hair follicles is never sufficiently affected by the vitamin to be observable either in gross appearance or in histological sections.

Materials and methods. These experiments were carried out in duplicate: in one set crystalline vit. A alcohol was used; in the other, vit. A acetate. Since the results were identical, no attempt will be made to describe or discuss them separately.

In the study of penetration, 10 mg of the

crystalline vitamin were dissolved in 2 ml of 95% alcohol, chloroform, oleic acid, linoleic acid or a paste made up of petrolatum, zinc oxide and talcum. After one topical application of vit. A dissolved in each of the above vehicles to the clipped skin of guinea pigs, biopsy punctures of skin were removed from each animal after 10 minutes, one hour, and 2 hours. In some cases, specimens were removed after 4 and 8 hours. The animals were maintained in a dark room to avoid inactivation of the vitamin by light. The skin samples were frozen without fixation. Sections were cut at 10 and 20 μ , mounted in glycerine, and studied under near-ultraviolet light filtered to transmit rays at 3600 Å. The penetration of vit. A can be traced by its brilliant fluorescence. Unlike the fluorescence of natural vit. A which fades quickly under ultraviolet light, that of the synthetic vitamin fades very slowly and is visible for about 20 minutes.

For the study of the effect of vit. A and of the vehicles alone on the skin, an aluminum ring $\frac{1}{4}$ " wide and $\frac{1}{2}$ " deep was fastened to the clipped backs of guinea pigs with methyl methacrylate and adhesive tape (Fig. 1, 2). The well was left empty overnight to make

* Crystalline vitamins used were obtained from the National Biochemical Co. and oleic and linoleic acids from the Fisher Scientific Co., both through the courtesy of the Desitin Chemical Co., of Providence, R. I.

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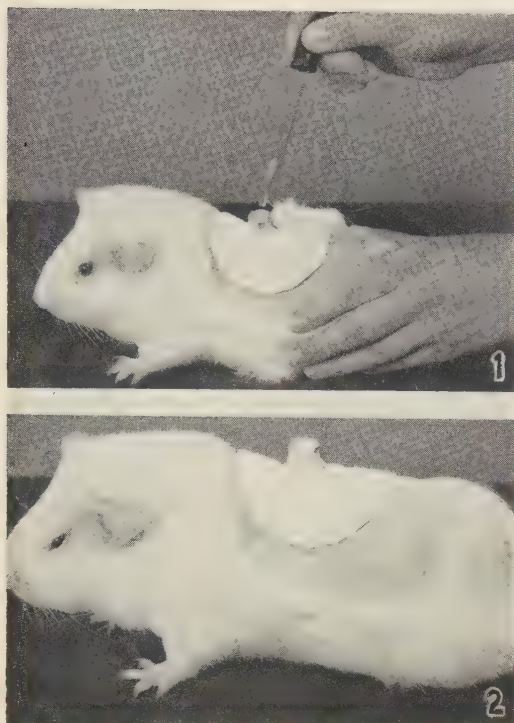


FIG. 1. Open aluminum well has been affixed to the skin with methyl methacrylate and steadied with adhesive tape. The well is ready for use.

FIG. 2. Well filled and closed with a fitted aluminum lid. A strip of adhesive tape over the lid prevents it from falling off.

certain that it had adhered properly. The next day the solutions of vit. A or of the vehicles alone were introduced into the well, which was then covered with a perfectly fitted aluminum lid. No leakage or spillage occurred. In this way the skin is in constant contact with the substances used and no other factors should influence the results. The following substances were used in groups of at least 6 animals: a) one milliliter of linoleic acid containing 5 mg vit. A; b) one milliliter of oleic acid with 5 mg vit. A; c) oleic acid alone; d) linoleic acid alone. Specimens of skin in contact with these agents as well as untreated control specimens from the same animal were removed after 3, 6, and 12 days. In another experiment the skin was treated twice daily with vit. A dissolved in 95% alcohol. At the end of 12 days a total of 20 mg had been applied to the area of skin within the well. Control animals received an equal amount of alcohol alone. Specimens of

skin treated with alcoholic vit. A and with alcohol alone were removed 6 and 12 days after the beginning of treatment. All specimens were cut into 2 pieces. One of these was fixed in Helly's fluid, the other in 1% trichloroacetic acid in 80% alcohol. Skin fixed in Helly's fluid was imbedded in paraffin and sectioned at 5 μ . For each specimen, alternate sections were stained with 0.05 toluidin blue buffered to pH 5.0(1), and with the periodic acid-Schiff method for glycogen(2). The material fixed in the alcoholic trichloroacetic acid was sectioned at 10 μ and the sections were used for the demonstration of sulphydryl and disulfide groups(3,4).

Observations. Penetration. The normal, untreated skin of guinea pigs shows little auto-fluorescence. In the epidermis, the Malpighian layer is non-fluorescent, and the keratin layer emits a pale yellow light of very low intensity. The dermis emits a blue-green light of low intensity, but the elastic fibers fluoresce with a strong white light. The hair follicles and sebaceous glands are non-fluorescent. White hairs emit a very pale green light, red or brown hairs fluoresce orange to red, and black hairs are non-fluorescent.

Ten minutes after the application of vit. A dissolved in alcohol, the stratum corneum and the sebum in the pilosebaceous orifices fluoresce with a bright greenish-yellow light. One hour after application, the fluorescence progresses to the sebaceous glands (Fig. 3, 4). After 2 hours the dermis around each sebaceous gland has small clouds of dust-like fluorescent particles. The subcutaneous fat, which is normally non-fluorescent, emits a pale greenish light.

Vit. A dissolved in chloroform penetrates even more rapidly and effectively than when dissolved in alcohol. Ten minutes after application, fluorescence can be seen in the stratum corneum of the epidermis, in the pilosebaceous orifices, and in the sebaceous ducts. After one hour, the stratum spinosum of the epidermis becomes fluorescent as do nearly all of the cells of the sebaceous ducts and glands. In resting hair follicles the fluorescence extends to the club, but in growing ones it does not pass deeper than the sebaceous

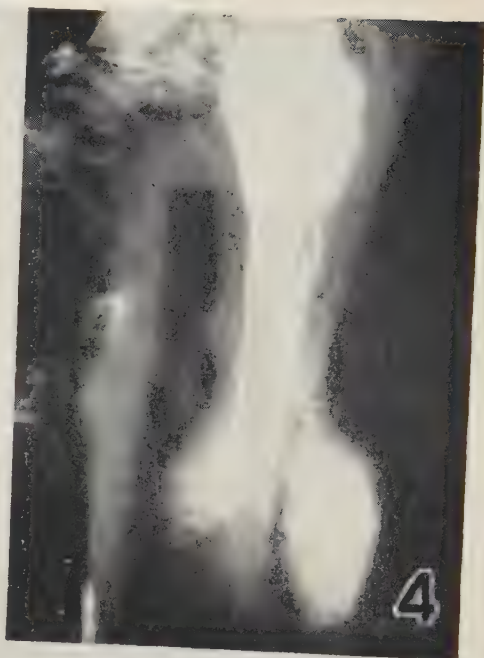


FIG. 3. Section through the skin one hr after application of vit. A dissolved in alcohol, viewed under near-ultraviolet light. Vit. A fluorescence is striking in stratum corneum, pilosebaceous ducts and sebaceous glands. Fluorescence of the hair is not due to vit. A but to a red pigment.

FIG. 4. Vit. A fluorescence in sebaceous glands and ducts one hr after application of vit. A dissolved in chloroform.

FIG. 5. Hypertrophied epidermis after one wk of contact with vit. A dissolved in oleic acid. Observe relatively thick stratum corneum. Stained with hematoxylin and eosin.

FIG. 6. Normal guinea pig epidermis stained with hematoxylin and eosin.

duct. After 2 hours there is some fluorescence in the dermis and in the subcutaneous fat.

Penetration of vit. A in oleic acid is very slow. At the end of 2 hours the vit. A fluorescence can be seen only as far as the sebaceous ducts. After 4 hours the sebaceous glands show a small amount of fluorescence. With linoleic acid, penetration is more rapid. By 2 hours the sebaceous glands show abundant vit. A fluorescence. Linoleic acid itself is slightly fluorescent, but the addition of the vitamin increases its fluorescence considerably. Penetration of vit. A dissolved in the viscous petrolatum paste is very slow. After 2 hours fluorescence was limited to the stratum corneum of the epidermis and to the sebum in the sebaceous ducts. Fluorescence appeared in the sebaceous glands after 4 to 8 hours.

Effect of vit. A on the skin. When in contact with vit. A dissolved in oleic or linoleic acids, the epidermis becomes progressively thicker (Fig. 5) and after 12 days it attains a marked hypertrophy. The stratum granulosum, which is normally one or 2 layers in thickness (Fig. 6), becomes thicker and the individual keratohyalin granules become larger. Many of the cells in the Malpighian layer are enlarged and misshapen, others are shrunken, with pycnotic nuclei, indicating cell death. The stratum corneum becomes somewhat thickened but it does not keep pace with the hypertrophy of the epidermis. Hair follicles and hair growth show no visible effect even after 12 days.

Histochemical preparations of skin kept in contact with vit. A in oleic or linoleic acid reveal a pronounced diminution in cytoplasmic basophilia (ribonucleic acid), particularly in skin treated for 12 days. Many of the cells in the upper part of the stratum spinosum become laden with glycogen. Glycogen is never present in the normal epidermis of the guinea pig. Also, there is a qualitative drop in the amount of sulfhydryl and disulfide content of the epidermis. The distribution of cytoplasmic basophilia, glycogen, and sulfhydryl and disulfide groups in the hair follicles is normal.

Oleic and linoleic acids used alone produce changes which are identical histologically and histochemically with those just described.

There is greater damage to the epidermis when oleic acid is used.

After the use of vit. A dissolved in alcohol, the epidermis also becomes hypertrophied. Cell distortion and necrosis, however, is minimal. The histochemical tests show no diminution of cytoplasmic basophilia and sulfhydryl and disulfide groups and no accumulation of glycogen. Skin treated with alcohol alone shows only a slight hypertrophy of the epidermis. Hair follicles and hair growth are not altered by either the alcoholic solution of vit. A or by the alcohol alone.

Discussion. The speed of penetration of vit. A through the intact skin is dependent upon the vehicle used. The vitamin penetrates most rapidly when it is dissolved in alcohol or chloroform. When dissolved in linoleic acid, in oleic acid, and particularly in the viscous petrolatum paste it penetrates more slowly. The avenue of entry of these substances, like that of carcinogens, is by way of the pilosebaceous canal and the sebaceous glands(5-7). What is being measured at first is the penetration of the vehicle with vit. A serving as an indicator. The spreading of vit. A fluorescence to the dermis and the subcutaneous fat is evidence that the vitamin penetrates beyond the sebaceous glands.

Application of large amounts of vit. A dissolved in oleic or linoleic acid to the skin causes an hypertrophy of the epidermis. The hypertrophy is not a specific effect of vit. A, since the oils alone induce similar effects(8). That the vitamin, however, can stimulate epidermal hypertrophy can be seen when it is applied dissolved in alcohol, for alcohol alone produces only mild hypertrophy. The hypertrophy caused by the vitamin dissolved in oils, or by the oils alone, is accompanied by a certain amount of epidermal damage. Damaged epidermal cells contain stored glycogen whereas undamaged ones do not(9,10). The hypertrophy which follows the application of the vitamin dissolved in alcohol is not accompanied by the storage of glycogen or by other visible epidermal damage.

It is agreed that vit. A inhibits or prevents keratinization(11,12). Large amounts of vit. A are said to have a local, non-specific action

on epidermal cells by interfering with the sulfhydryl metabolism of the epidermis(13, 14). Large doses of vit. A profoundly inhibit keratinization in the tissues of chick embryos grown *in vitro*(15). In the guinea pig local contact with oleic or linoleic acid, with or without vit. A, impairs keratinization of the epidermis only partially as seen by the relatively thick stratum corneum in Fig. 5. Keratinization is unaffected by the vitamin dissolved in alcohol. As the epidermis increases in thickness, the stratum granulosum also becomes thicker. This has been interpreted as an indication of impaired keratinization(12), although there is no evidence that increased production of keratohyalin indicates impairment of keratinization.

Treatment of skin for 6 days or longer with vit. A dissolved in oleic or linoleic acids, or with the oils alone brings about a diminution in histochemically demonstrable sulfhydryl groups. Since massive doses of vit. A dissolved in alcohol do not show this, the decrease in sulfhydryl groups, and thus keratinization, seems to be caused by the oil vehicle.

Vitamin A is said to cause hair loss in the mouse, rat, and rabbit(13,14,16). Yet, regardless of the amount of vit. A or of the type of vehicle used in the present experiments, the skin of the guinea pig shows neither impairment of hair growth nor hair loss. Resting hair follicles undergo some cellular hypertrophy similar to that of the epidermis and may cast off their club hairs, but growing follicles remain unaffected for the 12-day duration of the experiment. Lack of penetration cannot account for this, since, as we have seen, vit. A penetrates the skin readily.

Differences among the investigators may be due to a lack of appreciation of the biology of skin. For example, the "atrophy" of hair follicles, referred to in studies of vit. A deficiency, is not atrophy but quiescence of hair follicles. The "*hypoplasia of hair bulbs*" describing the inhibitory action of vit. A and similar unsaturated compounds on hair growth (16) has been illustrated by photomicrography of club hairs which must have come from quiescent hair follicles. Hair follicles have cycles of growth and rest, and the morphology

and physiology of the whole skin undergoes corresponding changes(17). The skin responds to injurious agents differently under these different conditions(18,19).

Summary. Vitamin A dissolved in alcohol or chloroform penetrates the intact skin quickly by way of the sebaceous glands. Dissolved in linoleic or oleic acid, or in a viscous petrolatum paste, it penetrates more slowly. Vit. A dissolved in oleic or linoleic acid or in alcohol causes an hypertrophy of the epidermis, but a similar hypertrophy is brought about by the fatty acids alone. Whereas the fatty acids, with or without vit. A, seem to retard keratinization slightly, the vitamin dissolved in alcohol does not. Hair growth was unimpaired even when the skin had received massive doses of vit. A dissolved in alcohol.

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Method of Obtaining Direct-Body Displacement-Velocity-Acceleration Ballistocardiograms of the Dog.* (21198)

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Animal experimentation was an essential phase in the development of electrocardiography and other cardiovascular methods, but as yet has contributed little to the study of ballistocardiography. Relatively limited animal experimentation has been conducted with the ballistocardiographic tables(1-3), and there have been no reports using the direct-body technic. This paper presents a modification of a human, direct-body technic(4), by means of which displacement, velocity and acceleration ballistocardiograms of dogs may be obtained. This technic permits the determination of ballistocardiographic alterations produced by drugs and by physiologic maneuvers under conditions not possible in the human.

Methods. Anesthesia was obtained either by a combination of morphine sulfate 2 mg/kg, subcutaneously, with urethane 1 g/kg, intraperitoneally, or by morphine sulfate 10 mg/kg, subcutaneously, with sodium pentobarbital 10 mg/kg, intravenously. Supplemental doses were administered when necessary. A concave aluminum rod was sutured to the spinous processes of 4 or 5 vertebrae between T-6 and L-5. The rod was made by transversely sectioning aluminum tubing of an inside diameter of 10/16 in. and an outside diameter of 12/16 in. The length of the rod was sufficient to extend at least 6 inches from the sacrum. The weights of the rods used in this study ranged from 40 g to 60 g, depending upon the size of the animal. The suturing of the rod to the spinous processes was easily accomplished by penetrating the skin, subcutaneous tissues and bone with a 15-gauge hypodermic needle and threading heavy cord through the needle. The animal was placed in the prone position in a wooden trough padded with 2-inch thick sponge rubber; the head was supported and the entire vertebral column was maintained in



FIG. 1. Position for obtaining ballistocardiograms of anesthetized dog. Animal is shown intubated for positive-pressure administration of oxygen by a rhythmic interrupter. Aluminum rod sutured to dog's spine is maintained in continuous contact with sensing unit of the ballistocardiograph.

a straight line relation (Fig. 1). The front legs of the animal were placed under the head support, extended, but not restrained. The back legs hung loosely over the end of the dog board or were folded under the animal. Additional padding was added if necessary to keep the spine parallel to the floor. The animal was intubated and respired with oxygen by a rhythmic motor-driven interrupter connected to a constant pressure tank. The combination of morphine and urethane anesthesia produced excellent skeletal muscle relaxation and depressed the natural respiratory movements without depressing vagal tone. Lighter grades of anesthesia were produced with pentobarbital and morphine. The respirator was stopped during ballistocardiographic recordings leaving the lungs stationary in the expiratory position. The animal usually made no respiratory movements during the few seconds required for recording, but if such movements persisted, they were eliminated by a short period of hyperventilation.

This method may be modified for the recordings of ballistocardiograms of the unanesthetized dog. Segments of nylon cord were inserted through each of several spinous processes, under pentobarbital anesthesia, and left indwelling. On subsequent days, the aluminum rod was tied to the spine without eliciting pain. Ballistocardiograms were obtained in this way as late as 2 weeks after the introduction of the nylon cord. The use of unanesthetized animals, while more physiological, in-

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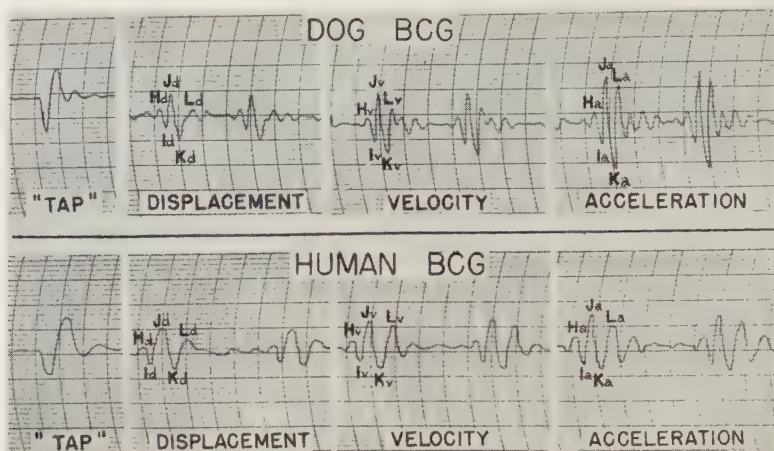


FIG. 2. *Upper tracings.* "Tap record," and displacement, velocity and acceleration ballistocardiograms of anesthetised dog. Recordings made 2 days after a strain gauge arch for measuring heart contractile force was sutured to right ventricle. *Lower tracings.* Ballistocardiograms of unanesthetised 27-year-old male with normal cardiovascular system. Dog and human tracings obtained during expiration. Vertical lines correspond to 0.2 sec. intervals.

troduces considerably more difficulty in the interpretations of recordings because of the artefacts of respiratory and voluntary movements. Accordingly, such recordings are satisfactory only with synchronous electrocardiographic recordings to aid in identification of ballistocardiographic complexes.

The Arbeit d-v-a ballistocardiograph(4)[†] was used to measure the displacement, velocity and acceleration of the animal's body movements. With each movement of the body, the rod sutured to the spine displaces the plunger of the sensing unit of the ballistocardiograph. The frequency response of the ballistocardiograph is better than 95% accurate from 2 to 20 cycles/second. The exclusion of higher frequency components is achieved through the use of an electronic low-pass amplifier. This is essential in order to eliminate artefacts due to ambient vibrations. Amplification, in the present experiments, was carried out by means of a direct-writing E.P.L. Cardiotron electrocardiograph. In several experiments, the output from this instrument was connected to a Brush B1-202 oscillograph. This oscillograph has recording speeds of 5, 25, and 125 mm/sec., and its 2 channels permit paired synchronous recordings of either ballistocardiograms, arterial

pressures, electrocardiograms, or heart contractile force changes by a strain gauge method(6). The frequency response of the Cardiotron-Brush system was found to be flat over the entire experimental range by use of a Hewlett-Packard Low Frequency Function Generator (Model 202A). The natural frequency of the dog was determined by tapping the animal quickly on the head while the ballistocardiograph selector dial was set on "displacement" and the amplification of the electrocardiograph was greatly reduced to eliminate the recording of ballistocardiograms. Such a record is shown in Fig. 2. The natural frequency of the dog, in the position described above, was found to range from 4 to 8 cycles/sec. If the natural frequency of the animal was above 5 cycles/sec., it was lowered by increasing the thickness of the sponge rubber padding. The degree of damping as observed on the tap records ranged from about 0.3 of critical to nearly critical. The final natural frequency and degree of damping of the dog, positioned in this manner, approximates values found in the human. The light weight of the rod and its secure attachment to the spine insures faithful following of the body movements by the rod. In this respect, the dog ballistocardiogram has higher fidelity than that of the human, where the record is obtained from a shin-piece applied to the skin

[†] Manufactured by the Industrial Development Laboratories, Jersey City, N. J.

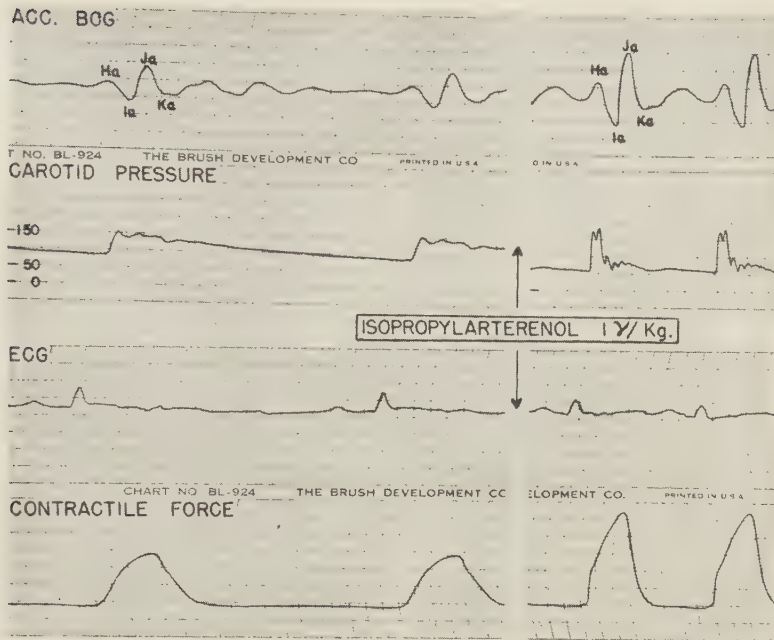


FIG. 3. High speed oscillographic recordings of acceleration ballistocardiogram, carotid blood pressure, electrocardiogram (LII), and heart contractile force of anesthetized dog before and after intravenous administration of isopropylarterenol. Scale on B.P. tracing indicates mm Hg. Heart contractile force proportional to excursion amplitude. Tracings obtained 2 days after a strain gauge arch was sutured to right ventricle. Vertical lines correspond to .04 sec. intervals.

with its underlying layers of elastic media. The choice of displacement, velocity or acceleration recordings in dog ballistocardiography involves essentially the same factors as in the human(4,5). The mathematical process of differentiation from displacement to velocity to acceleration results in an emphasis of high frequency components and a minimizing of low frequency waves. If the amplitude of response of a linear physical system to an input of sinusoidal force of constant amplitude is plotted with respect to the ratio, Forcing Frequency/Natural Frequency, it is found that only those frequency components below the natural frequency of the body are accurately measured by displacement curves, whereas higher frequency components can only be obtained by acceleration recordings. Since the natural frequency of the dog was lowered to approximate values found in the human, and since almost all of the disturbing forces seen were of a higher frequency than the natural frequency, acceleration recordings were almost exclusively used in the present study. Without anesthesia, only acceleration

recordings could be obtained because displacement and velocity tracings were distorted by the low frequency respiratory and skeletal muscle movements.

Results. Preliminary experiments(7) with the Dock electromagnetic ballistocardiograph (8) yielded tracings which were an indeterminable mixture of displacement and velocity and the use of this instrument was discontinued.

The tap record, and displacement, velocity and acceleration ballistocardiograms of an anesthetized dog are illustrated in Fig. 2. The wave components and contours are similar to those obtained with the human as may be noted on comparing those tracings with the recordings in the lower part of the illustration.

Fig. 3 illustrates the effects of 1 γ /kg of isopropylarterenol, intravenously, on the synchronously recorded acceleration ballistocardiogram, electrocardiogram, heart contractile force(6) and carotid pressure of an anesthetized dog. The ballistocardiographic effects produced by isopropylarterenol in the dog are similar to those observed in the human by

Kaufman, Iglauer, and Herwitz(9). As may be noted in Fig. 3, the Ia-Ja amplitude of the acceleration ballistocardiogram increased by an increment of 100% of control values; heart contractile force increased by approximately the same increment. The Ia-Ja time was reduced from .04 to .02 sec.; the total time of the heart contractile force curve decreased from .23 sec. to .14 sec. There was a 30 mm decrease in diastolic pressure and a 40 mm increase in systolic pressure. The heart rate increased from about 85 beats/min. to 220 beats/min. The increased Ia-Ja amplitude and contractile force along with shortening of the Ia-Ja time and the curve of contractile force change are consistent occurrences with this drug. The relation of these changes and those of arterial pressures and heart rate is being studied. A preliminary report(10) has been published describing changes produced by other sympathomimetic amines in the dog and human acceleration ballistocardiogram.

Summary. A method of obtaining direct-body displacement, velocity and acceleration ballistocardiograms of the dog has been de-

scribed. Ballistocardiograms obtained by this method are similar to those of the human.

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Stability of Vesicular Stomatitis Virus at Varying Hydrogen Ion Concentrations. (21199)

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Various workers(1-5) have studied the stability of vesicular stomatitis virus at varying hydrogen ion concentrations. The detailed investigations of Galloway and Elford(5) have demonstrated the loss of viral infectivity at approximately pH 4.0 and its persistence at pH 9.6. Their criterion for survival of infective virus consisted of presence of characteristic lesions in the foot pads of guinea pigs inoculated intradermally with virus. No information is given on the stability of virus beyond pH 9.6 and no quantitative data are available concerning the actual number of infective viral particles surviving at any given hydrogen ion concentration. The lack of adequate quantitative data as well as the pos-

sible consequences associated with the common practice of disinfection of infected materials with sodium hydroxide emphasized the need for reexamination of this problem. The present studies utilizing more sensitive experimental hosts (mice and embryonated eggs as opposed to guinea pigs) in viral infectivity tests, report quantitative data on the survival of 2 strains of vesicular stomatitis virus over a wide range of pH.

Materials and methods. The viruses used in these studies were the New Jersey and the Indiana strains of vesicular stomatitis. These strains had histories of passages in various experimental hosts; the most recent passages were made in embryonated eggs via the al-

lantoic route. Infected allantoic fluid was used as a source of virus. The pH of the viral suspension was adjusted either by dilution (1:10) of virus-containing allantoic fluid in an appropriate buffer of desired pH or by addition of 0.5 N HCl or NaOH until the desired pH was achieved. These samples were left for 1 hour at room temperature (25°C), and the pH of the suspension was determined electrometrically at the beginning and at the end of the hour period. Determination of infectivity was made by a) intracerebral inoculation of 0.03 ml of serial dilutions into each of a group of 6 to 8 Namru(6) mice 4 to 6 weeks of age or b) suballantoic inoculation of 0.1 ml of serial dilutions into each of a group of eight 9-day embryonated eggs. All dilutions of virus were made in 2% peptone solution. Mice were observed daily for a period of 10 days and deaths were recorded. Inoculated eggs were examined daily for deaths over a period of 4 days. Whenever there was any doubt concerning the viral origin of death of embryos, the allantoic fluids were tested for specific antigen in the presence of vesicular stomatitis antiserum. The 50% mortality endpoint was calculated by the method of Reed and Muench(7).

Results. Table I summarizes the results obtained with mice and embryonated eggs of the effect of pH upon the stability of the infective component(s) of vesicular stomatitis virus. The data shown in the table are representative of the results obtained for both virus strains in a number of similar experiments.

The data indicate that the infectivity of both strains of viruses for mice is not markedly affected by acidic reactions until a pH value of 4.0 or less is employed. No infective virus was demonstrable after exposure of virus at pH 2.0 for 1 hour at room temperature. Virus appears extremely resistant to inactivation in alkaline environments. There was no great change in infective titer of the New Jersey strain up to pH 10.4 and only a slight decrease at pH 11.6. Marked destruction of viral infectivity occurred, however, upon exposure of virus to a pH value of 12.5. The results of infectivity tests in mice with the Indiana virus were quite similar, although this strain seemed to be slightly more sus-

TABLE I. Effect of pH upon Infectivity of Vesicular Stomatitis Virus.

pH	Buffer-system	Virus titer*			
		New Jersey strain		Indiana strain	
		Mice	Eggs	Mice	Eggs
7.2	Peptone broth	5.9	5.8	5.3	5.6
2.0	KCl-HCl	<1.0	<1.0	<1.0	<1.0
3.0	Citrate-HCl	2.3	2.5	1.0	2.3
4.0	Acetic acid-acetate	3.7	2.6	2.1	3.0
5.0	<i>Idem</i>	5.0	4.8	4.5	4.4
6.0	KH ₂ PO ₄ - NaOH	5.5	5.7	4.7	5.2
7.0	<i>Idem</i>	5.8	5.8	5.3	5.7
8.0	"	6.3	6.0	5.2	4.7
8.9	H ₃ BO ₃ - KCl - NaOH	5.7	5.4	5.0	5.1
9.7	<i>Idem</i>	6.2	5.8	5.4	4.0
10.4	Glycine - NaOH	6.2	5.1	4.2	3.5
11.6	<i>Idem</i>	4.1	3.4	1.6	<1.0
2.0	pH adjusted with HCl	No virus	—	—	—
12.5	pH adjusted with NaOH	<1.2	—	—	—

* Expressed as log LD₅₀ remaining.

— Indicates sample not tested.

ceptible to inactivation at alkaline reactions. It is evident from Table I that the results obtained with embryonated eggs closely parallel those with mice.

Discussion and Summary. The present results are in conformity with the data of Galloway and Elford(5) concerning the instability of vesicular stomatitis virus at pH values of 4.0 or less. The use of more sensitive experimental hosts and determination of 50% mortality endpoints, however, showed that inactivation of virus was achieved only at pH 2.0. Extension of studies beyond the pH value of 9.6 reported by Galloway and Elford (5) revealed the striking resistance of virus to inactivation by alkaline environments. It would appear that the wide range of stability of vesicular stomatitis virus may render it a likely prospect for purification by means of the ion exchange resins.

Grateful acknowledgement is made to Miss Jane Seely and Miss Doris Clinger for their valuable technical assistance.

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Sexual Development in Female Rats Treated with Cortisone.* (21200)

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An excess of endogenous adrenal oxysteroids as found in Cushing's Disease is associated with sexual dystrophy(1). Hench *et al.*(2) found that adolescents treated with cortisone suffer menstrual disturbances more frequently than adults. While cortisone treatment in women with regular menstrual cycles leads to irregularities, it may lead to establishment of menses in amenorrheic individuals(3). The observation of Sohval and Soffer(4) and of Maddock *et al.*(5) that patients on cortisone excrete excessive amounts of gonadotrophin suggests that these menstrual changes may be related in part to alterations in pituitary function and that cortisone may stimulate the production of gonadotrophin. Antopol(6) found that the ovary and accessory reproductive organs of immature mice treated with 1.25 mg of cortisone weighed less than normal. Byrnes and Shipley(7) found that Lipo-adrenal Cortex and Adrenal Cortex Extract inhibited gonadotrophin secretion in ovariectomized females in parabiosis with an immature female while daily doses of 0.5 or 2.0 mg of cortisone were ineffective. Fawcett and Deane(8) found no difference in water content or stroma development of uteri of ovariectomized rats treated with cortisone and/or estrogen. However, Szego(9) found a decreased hydration of uterus 4 hours after intravenous injection of estrogen to cortisone-treated rats, but no depression of wet weight. Talalay(10) reported that 2.5 mg of cortisone daily for 3 days partially prevented the increase in uterine weight produced by estrogen in ovariectomized rats. With administration of 0.5-5.0 mg of cortisone

daily to young rats, Moore(11) found an increase in weight of the ovary accompanied by variable changes in uterine weight. It appears, therefore, that there is no agreement among investigators on the effects of cortisone on the female reproductive system and that only limited information is available on its effects on production and release of pituitary gonadotrophin.

The purpose of this investigation was to study the effects of a small dose of cortisone on the development of the reproductive system and on the gonadotrophic hormone content of the hypophysis of immature female rats.

Materials and methods. Four groups of 23-day-old female rats, 6 pairs to each group, were pair-fed with Rockland Milled Stock Diet. Another 4 groups of 23-day-old female rats, 8 pairs to each group, were fed *ad libitum*. One member of each pair was treated daily with 1 mg cortisone acetate[†] subcutaneously. At the end of 2 and 4 weeks of treatment as well as 3 weeks after cessation of treatment, 6 or 8 pairs of rats, respectively, were autopsied. Tissues were fixed in Bouin's solution or alcoholic-formalin and stained with hematoxylin and eosin or periodic acid-leucofuchsin, respectively. The pituitary of each animal was frozen individually for subsequent gonadotrophin assay. The uteri of 3 pairs of animals in each group were dried to constant weight at 110°C to determine their water content.

Results. Body weight gain was 22.8 to 48.0% less in the treated animals than in the controls. With cessation of treatment, the

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[†] Cortisone acetate was generously supplied by Doctor E. Alpert of Merck and Co.

TABLE I. Reproductive System in Pair-Fed Female Rats* Treated with Cortisone.

Treatment, wk	Ovary mg/100 g	Uterus drained body wt	Days of vaginal opening				
			36	38	40	42	46
2	47.1	110.6	1				
C†	22.2	71.5	3				
4	32.5	195.9	0	0		1	6
C	18.9	61.8	0	4		6	6
2 + 3§	38.6	209.8	0	3	6		
C	30.1	122.6	4	6	6		
4 + 3§	40.5	201.3	0	1	4		6
C	29.7	112.3	3	4	5		6

* 6 pairs in each experiment.

† 1 mg cortisone acetate daily starting at 23 days of age.

‡ C = Controls.

§ Autopsied 3 wk after cessation of treatment.

|| P = <.001.

animals resumed an increased rate of growth, so that the final weight of the treated animals sacrificed 3 weeks after cessation of treatment was only 6.8 to 33.5% less than the weight of their controls.

Adrenal and thymus weights were 41.3 to 84.0% less in the treated rats. Three of the 4 groups of animals posted 3 weeks after cessation of treatment showed a 3.2 to 8.4% increase in adrenal weight over the controls instead of a decrease, indicating that during this period of recovery the adrenals had undergone marked growth. This is in agreement with the quick recovery from the inhibitory effect of cortisone on ACTH production and release and consequent adrenal growth noted by others(12). The thymus remained reduced in weight. The thyroid was 11.8 to 16.0% heavier in 3 of 4 groups in both the pair fed and *ad libitum* fed experiments. The effects of cortisone were, in general, more pronounced in the *ad libitum*-fed than in the pair-fed animals.

Reproductive tract. The ovaries of treated pair-fed animals were significantly ($P = <0.001$) heavier than those of the controls (Table I). The differences are greater in animals posted at the end of the treatment than in those sacrificed 3 weeks after treatment was discontinued. The uteri were heavier in treated than in non-treated animals, but only in the 3 older groups was the difference statistically significant ($P = <0.001$).

In *ad libitum*-fed groups, ovaries were slightly heavier in the treated animals killed at the end of the treatment than in the controls (Table II), but the differences were less marked than in the corresponding pair-fed groups. In animals sacrificed 3 weeks after termination of treatment, ovaries were significantly ($P = <0.001$) lighter. This is contrary to the results obtained in the pair-fed animals. Compared to controls, uterine weights were slightly less in rats treated for 2 weeks, but heavier in those treated for 4 weeks. This difference in response of the uteri may be due to the longer period of treatment.

Opening of the vagina was delayed in all groups (Tables I and II). The delay was greater in those treated for 4 weeks. When vaginal smears were made of the last group during the last week of the experiment, normal cycles were found.

Histological examination of ovaries revealed a greater degree of follicular development in treated animals than in the controls, due especially to increased size and number of the follicular antra. Three of the pair-fed animals autopsied after 4 weeks of treatment had several corpora lutea, but these were present in only 1 of the pair-fed controls. All rats in the older groups killed at 5 or 7 weeks after start of the experiment, and 3 weeks after cessation of treatment, had corpora lutea indicating that ovulation had occurred. Examina-

TABLE II. Reproductive System in *Ad Libitum*-Fed Female Rats* Treated with Cortisone.

Treatment, wk	Ovary mg/100g	Uterus drained body wt	Days of vaginal opening							
			30	33	36	37	38	40	45-47	
2	37.1	172.6	0	0			1			
C†	33.4	176.3	2	8			8			
4	39.5§	201.4				0		1	8	
C	36.4	186.9					2	8	8	
2 + 3	38.3	174.6§		0			1		8	
C	40.1	185.0		1			8		8	
4 + 3	39.9	218.1		0			1		8	
C	46.0	202.9		1			4		8	

* 8 pairs in each experiment.

† 1 mg cortisone acetate daily.

‡ C = Controls.

§ P = <.05.

|| Autopsied 3 wk after cessation of treatment.

¶ P = <.001.

TABLE III. Pituitary Gonadotrophin Assay of Pair-Fed Rats Treated with Cortisone.

Treatment, ‡ wk	Donor* Pituitary		Recipient† Ovary Uterus	
	mg/100 g body wt			
2	7.4§	30.4§	138.9	
Controls	5.8	28.4	123.7	
4	6.1§	28.5§	98.4§	
Controls	4.9	26.2	127.5	
2 + 3	5.3¶	30.5§	134.4	
Controls	4.5	28.5	123.5	
4 + 3	6.5§	27.1	64.3	
Controls	4.9	27.0	77.3	

* 6 pairs in each experiment.

† 23 days old at start of pituitary inj.

‡ 1 mg cortisone acetate daily starting at 23 days old.

§ $P = <.001$.

|| Autopsied 3 wk after cessation of treatment.

¶ $P = <.01$.

tion of the uteri showed no marked differences in the development of endometrial stroma or glands. Differences in water content of uteri of treated and control animals were less than 4%. This is in agreement with the observation of Fawcett and Deane(8) that in ovariectomized rats, simultaneous treatment with estrogen and cortisone did not significantly modify the effect of estrogen on the water content of the uterus. No significant differences were seen in the distribution and concentration of glycogen in the uterine tissue of treated and control animals.

Gonadotrophin content of pituitary. In an attempt to obtain information on the gonadotrophic activity of the pituitary, each frozen gland was homogenized in 3 cc of saline solution (pH = 8). One-half cc portions of the suspension of each gland were injected 2 times per day for 3 days into a 23-day-old female rat which was autopsied on the fourth day. Pituitaries of treated animals were heavier than those from corresponding controls (Tables III and IV). In Table III it is seen that pituitaries from treated pair-fed animals of all except the oldest group caused a significantly ($P = <.001$) greater development of the ovaries of the test rats than did the pituitaries of the controls. Ovaries of rats receiving pituitaries from treated animals showed more advanced follicular development as indicated by number and size of antra. Uterine weights were increased in animals re-

ceiving pituitaries from pair-fed animals treated for 2 weeks, but decreased when donors had been treated for 4 weeks. In recipients of pituitaries from treated animals fed *ad libitum*, ovaries were heavier in 3 of the 4 groups (Table IV). Uterine weights were increased in animals receiving pituitaries from *ad libitum*-fed donors autopsied at termination of treatment, but were decreased in those autopsied 3 weeks later. The gonadotrophic activity of the pituitary of treated rats was greater than that of the pituitary of pair-fed controls. This is indicated by 1) a generally heavier weight of the ovaries in the former, and 2) a greater development of the ovaries as indicated by the increased number and size of antra in animals receiving pituitaries from treated animals.

Discussion. A reduction in body, adrenal and thymus weights during treatment with cortisone has been previously reported(12,13). The marked growth of the adrenals after cessation of treatment confirms the findings of Patterson *et al.*(12) who found that the adrenals reattained normal histological appearance in 7-10 days after end of treatment.

With administration of 2-5 mg of cortisone daily to young rats, Moore(11) found an increase in the weight of the ovary and greater development of ovarian follicles similar to that observed in our experiments. This could be due to either an increased sensitivity of the

TABLE IV. Pituitary Gonadotrophin Assay of *Ad Libitum*-Fed Rats Treated with Cortisone.

Treatment, ‡ wk	Donor* Pituitary		Recipient† Ovary Uterus	
	mg/100 g body wt			
2	5.6§	31.9	181.2§	
Controls	4.9	28.4	100.0	
4	5.9§	31.3	81.7¶	
Controls	5.2	30.8	70.7	
2 + 3**	5.1	26.9¶	73.6§	
Controls	5.1	30.8	80.5	
4 + 3**	5.5§	28.3¶	103.7§	
Controls	5.2	26.2	130.8	

* 8 pairs of female rats in each category.

† 23 days old at start of pituitary inj.

‡ 1 mg cortisone acetate daily starting at 23 days of age.

§ $P = <.001$.|| $P = <.05$.¶ $P = <.01$.

** Autopsied 3 wk after cessation of treatment.

ovary to stimulation by gonadotrophic hormones or greater production of gonadotrophins under the influence of cortisone acetate. The increased incidence of corpora lutea in animals autopsied after 4 weeks treatment compared to controls suggests an increased production and/or release of both follicle-stimulating and luteinizing hormones of the hypophysis.

While in all of our pair-fed and in some of our *ad libitum*-fed groups, there was a marked increase in weight of the uteri of treated animals compared to controls on a mg per 100 g body weight basis, no such differences were reported by Moore(11). This may be due either to the differences in dosage and/or periods of treatment. Similarly, the report by Talalay(10) of a depression by cortisone of estrogen stimulation of uterine growth probably represents an effect of differences in treatment regime or experimental design.

Data obtained in pair-feeding experiments probably are more accurate than those obtained in rats fed *ad libitum*, since we found that animals treated with 1.0 mg cortisone daily consumed 30-50% less food than controls. The better uterine growth in *ad libitum*-fed controls is therefore due, in part, to their greater food consumption.

The greater weight of the uteri of pair-fed treated animals probably represents a quantitative increase in growth of the uterus in response to increased production of ovarian hormones by the hypertrophied gland. This hypothesis is supported by the lack of qualitative histological differences in development of endometrial stroma or glands, in water content or glycogen distribution between treated and control animals. Similarly, Fawcett and Deane(8) reported no difference in development of stroma or water content in ovariectomized rats treated simultaneously with cortisone and estrogen as compared to estrogen alone.

Although ovaries of treated donors show a greater degree of development, the opening of the vagina, a secondary sexual character, is delayed. This may indicate a decrease in sensitivity of the vaginal plate tissue to estrogen or a decreased production of estrogen by the ovary under the influence of cortisone. Since both the ovary and the uterus, which is dependent on estrogen for growth, are heavier

in the treated animals than in the controls, a decreased sensitivity of the vaginal plate to growth stimuli appears to be the more probable cause for the delay in its opening.

Gonadotrophin content of pituitaries, as determined by ovarian response, appears greater in rats treated for 2 or 4 weeks than in pair-fed controls. This difference declines as the animals grow older and is smaller in those autopsied 3 weeks after cessation of treatment. This decrease in the difference between controls and treated animals in the older groups may be due to a normal process of maturation of the animals resulting in an increased production of pituitary gonadotrophin accompanied by an increased ovarian hormone secretion. The data indicate that there is an increased production of gonadotrophin and not only an increased storage in cortisone-treated animals since such donor animals also show greater development of ovaries. The possibility that the altered development of the reproductive system after cortisone treatment may be the result of a modified metabolism does not appear likely in view of the fact that the glucose and non-protein nitrogen content in blood and urine samples taken at autopsy showed no significant differences from normal.

Our observations suggest that the increased weight and greater follicular development of ovaries in treated animals are due to the action of cortisone on the pituitary rather than on the gonads or reproductive tract. In nearly all cases, the pituitaries taken from animals with the heavier ovaries produced the greatest stimulation of ovaries in test animals. The lack of effect of cortisone on gonadotrophin secretion in parabionts reported by Byrnes and Shipley(7) may be due to the range of dosage employed.

Alterations in pituitary gonadotrophin may be due to the fact that cortisone, through its inhibition of ACTH, could reduce sex steroid production by the adrenal. The ability of exogenous cortisone to inhibit secretion of adrenal sex hormones has been shown by Wilkins *et al.*(14) and others. Decrease of adrenal sex steroid secretion may allow an increased production of pituitary gonadotrophin so as to stimulate gonadal growth and hormone secretion.

Summary and conclusions. 1. Immature female rats were treated with 1.0 mg of cortisone acetate daily for 2 or 4 weeks starting at 23 days of age and were maintained on pair or *ad libitum* feeding regimes. 2. Body weight, adrenal and thymus weights were reduced compared to control animals. These differences were less marked when animals were autopsied 3 weeks after cessation of treatment. 3. The weights of ovary and uterus were generally greater in the treated animals. The increased weight of the ovaries was due to a greater number of follicles and to an increased size of the antra. 4. Pituitary glands removed from animals treated with cortisone appeared to have a greater gonadotrophic potency than normal as indicated by the growth of the ovaries in immature rats injected with these glands. 5. Possible explanations for these changes are discussed.

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A Means of Increasing the Tolerated Dose of Isoniazid in Mice. (21201)

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(Introduced by B. J. Olson.)

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Isoniazid (INH) as a therapeutic agent in human tuberculosis is in widespread use; one limiting factor is its toxicity (1-5). Two well-known detoxifying agents, glycine and sodium glucuronate, administered simultaneously with the INH in mice, have shown a marked effect in increasing toleration to this therapeutic drug. Three types of experiments are reported here: 1) Survival of DBA mice (20-g weight) after single and repeated oral administrations of toxic amounts of INH with different quantities of the 2 chemicals; 2) blood plasma levels of INH after a single oral administration of the 3 chemicals; and 3) an

in vitro test of the effect of the detoxifying chemicals on the bacteriostatic action of INH on tubercle bacilli.

Inasmuch as the toxicity data were available for white mice only, a preliminary determination of the toxic dose of INH in DBA mice was necessary. According to Grunberg and Schnitzer (6) the LD₅₀ by gavage in white mice was found to be 203 mg/kg. In our laboratory this amount in white mice resulted in 57% survival, but in the DBA strain all the mice survived. However, an oral dose of 250 mg/kg (5 mg/20-g mouse) resulted in only 45% survival in a group of 49 DBA mice. In contrast, when 50 mg of sodium glucuron-

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TABLE I. Mouse Survival following Oral Administration of 8 mg Isoniazid with Sodium Glucuronate and Glycine. Single dose.

Sodium glucuronate, mg	Glycine, mg	Mouse survival (DBA strain)	
		No. survivors	% survival
		No. treated	
0	0	2/70	3%
5	0	0/20	0
10	0	0/20	0
25	0	1/10	10
50	0	2/20	10
75	0	10/20	50
0	5	0/20	0
0	10	1/20	5
0	25	5/10	50
0	50	13/20	65
0	75	13/20	65
5	5	1/15	7
5	25	6/10	60
10	10	3/15	20
10	25	10/10	100
20	25	10/10	100
25	5	3/10	30
25	10	3/10	30
25	20	8/10	80
25	25	63/65	97
50	50	9/10	90
75	75	20/20	100

ate monohydrate[†] were added to the 5 mg of INH per mouse the survival of the 8 mice tested was 100%.

Mortality after a single administration of chemicals. In view of this preliminary observation with sodium glucuronate, a larger dose of INH, 8 mg (400 mg/kg), which alone had proved to be 100% lethal within 2 hours, was tested in combination with several concentrations of sodium glucuronate. At the same time, the effect of various amounts of glycine with and without the glucuronate was studied. The chemicals were weighed, combined in one tube, and dissolved in water to such a volume that the desired concentration of each was contained in 0.5 ml for oral administration. At least 10 DBA mice were used for each test solution. The data recorded in Table I include the results of a series of tests with the 3 chemicals in various proportions. Here it is seen that, whereas with 8 mg of INH alone

only 2 of the 70 mice survived, the addition of glucuronate or glycine increased the survival rate. The optimum results were found when both chemicals were used. For with 25 mg of each chemical 97% of a group of 65 mice tolerated the 8 mg toxic dose. The smallest amounts of these chemicals necessary for 100% survival of 10 mice with the 8-mg dose of INH were found to be 25 mg of glycine and 10 mg of sodium glucuronate. In other words, the use of equimolar amounts of INH and sodium glucuronate plus 5 or 6 moles of glycine will enable the mouse to withstand an amount well above the minimal lethal dose of INH alone (6 mg/20-g mouse).

Mortality after repeated administration of chemicals. Two experiments show the mouse survival time following repeated administrations of a lethal dose of INH mixed with excessive amounts of glycine and glucuronate. In the first experiment, each of 20 DBA mice was given a mixture of 8 mg INH with 75 mg of sodium glucuronate and 75 mg of glycine on 5 successive days. The first toxic fatality in these mice occurred after the third dose and first outward symptoms of drug accumulation were apparent when a few animals became hyperactive and 4 succumbed after the fourth treatment. After the fifth and last daily dose all were hyperactive and only 4 mice survived more than 2 hours. Increased tolerance was definite inasmuch as only 2 of the 70 control animals survived one dose of 8 mg INH alone, and 95% of the treated animals survived 3 daily doses of the mixed chemicals. In the second experiment, with 10 mice for each dose, INH in concentrations of 10, 15, 20, 25, 30, and 40 mg was combined with the detoxifying chemicals in the amounts shown in Table II, and administered orally at 72-hour intervals. The treatments were continued until approximately 50% mortality was reached. Under these conditions, the 10 mg dose of INH could be tolerated for 15 times during a period of 45 days, for, with 2 accidental losses, only one mouse of the 10 succumbed to the drug after the 14th treatment. With 15 mg in each treatment, a 50% endpoint occurred after 5 doses, and with 20 mg, after 2 doses. Indeed it is amazing that, even with 30 mg of INH mixed with these

[†] Kindly supplied by the Corn Products Refining Co., New York.

TABLE II. Mouse Survival following Oral Administration of Isoniazid with Sodium Glucuronate and Glycine. Repeated administration at 72-hr intervals.

Isoniazid, mg	Sodium glucuronate, mg	Glycine, mg	Survivors of 10 DBA mice after treatments														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10	50	50	10	10	10	10	10	9*	9	9	9	9	9	9	8*	7	7
15	50	50	10	10	7	6	6	4	2								
20	75	75	10		4												
25	100	100	10	6													
30	125	125	4														
40	150	150	0														

* One mouse missing for 6th treatment; one killed accidentally during 13th treatment.

detoxifying chemicals, 4 of the 10 mice survived one dose, and only when the single dose was as much as 40 mg—more than 6 times the usual lethal amount—did the mortality reach 100%.

Blood plasma levels. Blood plasma levels were studied to ascertain the absorption of larger doses of INH in the presence of these additional chemicals. Each of 45 DBA mice was given orally a solution containing in 0.5 ml 8 mg INH, 25 mg sodium glucuronate, and 25 mg glycine. In groups of 15 the mice were sacrificed at 24, 48, and 72 hours after drug administration and the hearts' bloods from each group pooled. Assay of INH in the blood plasma was carried out by the Rubin method(7) and the "pyridyl" method (Prescott, Kauffmann, James(8)). For controls, blood plasma levels were assayed in the pooled bloods of 10 mice 24 hours after each was given 4 mg of INH, the maximum amount which permits 100% survival of DBA mice for this length of time. It was found that the control (4 mg) plasma yielded 0.13 mg % INH. On the other hand, the plasma from the mice given the 8 mg INH mixture, after 24 hours yielded 0.88 mg %, a 6-fold increase over the control; after 48 hours 0.45 mg %, and after 72 hours 0.18 mg %, approximately like the control at 24 hours. In 2 other tests, whereas the control (4 mg) plasma levels were 5.5 and 5.2 mg % at one hour and negligible at 24 hours, after a 10 mg dose of INH with 50 mg each of glycine and glucuronate, the plasma levels were 6.4 and 6.2 mg % at one hour, and 0.7 and 0.8 mg % at 24 hours. Thus, at least in DBA mice, appreciably higher plasma levels were maintained for a longer time following large doses of INH in

combination with sodium glucuronate and glycine than with the 4 mg dose of INH alone.

Effect on bacteriostatic action of INH. One procedure has been employed to determine whether the presence of either sodium glucuronate or glycine, or both, has any appreciable effect on the *in vitro* bacteriostatic action of INH on tubercle bacilli. One mg of INH alone or in combination with the chemicals, in the proportions effective *in vivo*, was dissolved in 0.5 ml volume, and added to 9.5 ml of horse meat infusion broth. Dilutions were made in broth. The tubes were inoculated with 0.2 ml of a 6-day growth of the human strain B103 of tubercle bacilli and incubated at 37°C for 6 days. Macroscopic readings of growth were recorded on the third and sixth days. The inhibition endpoint of INH alone or in mixture was 0.01 mg/ml. The control sodium glucuronate or glycine in the doses used had no effect on this culture. Hence it may be concluded that these 2 chemicals, effective as detoxifying agents *in vivo*, were without effect on the bacteriostatic action of INH with this strain of tubercle bacilli.

Discussion. The metabolic changes involved in this detoxication are not at present established. Presumably the toxic manifestations following the use of INH are largely due to an imbalance in normal metabolism, possibly a blocking or exhaustion of normal detoxifying agents(9-11). The introduction of supplementary quantities of the 2 chemicals, glycine and sodium glucuronate, both of which are normally available in the body, permits mice to survive an amount of INH some 5 times larger than the otherwise toxic dose. The fact that the results are better when both chemicals are used, than with

either alone, suggests that the INH may be split into toxic products which are separately conjugated with the respective chemicals either directly or by enzyme action.

The recent report of Ragno and others(12), suggests the explanation that the amino acid combines with ammonia liberated from the INH. These investigators have used glutamic acid as a detoxifying agent with INH in patients with tuberculous meningoencephalitis. With such combined therapy they have been able to use larger oral doses of INH without toxic disturbances, and have obtained higher concentrations of the drug in the spinal fluid.

Summary. Attempts have been made to find a means of increasing the amount of isoniazid (INH) which mice will tolerate. In the DBA strain of mice, INH alone was lethal for 45% of the animals when 5 mg/20 g mouse (250 mg/kg) were given orally. With an 8 mg dose of INH, lethal *per se*, simultaneous administration of as little as 25 mg glycine and 10 mg sodium glucuronate monohydrate was sufficient to keep alive all the animals tested. By the use of appropriate amounts of the 2 detoxifying chemicals, all mice survived either a single oral dose of 25 mg INH, or 3 doses of 8 mg daily, or 13 doses of 10 mg each given at 72-hour intervals. Larger amounts were not tolerated. A study of the blood plasma concentrations of INH showed that, whereas 24 hours after a 4-mg dose alone only a negligible amount remained,

appreciably higher concentrations, approximately 6-fold, persisted for a longer period following 8 or 10 mg INH in combined treatment. In the proportions tested, sodium glucuronate and glycine had no effect on the bacteriostatic action of INH *in vitro* on one human strain of tubercle bacilli.

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Nitrogen Requirements of *Glaucoma scintillans* and *Colpidium campylum*.*

(21202)

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The ciliated protozoans, *Glaucoma scintillans* and *Colpidium campylum* were isolated and established in axenic (bacteria-free) cul-

ture in 1941(1). They were grown in a medium containing yeast cell fragments, and because growth failed when the particles were withheld it was concluded that they were phagotrophic. Later Peterson(2) was able to grow *Colpidium* in a non-particulate medium containing yeast protein fractions. We have found that *Glaucoma* also is dependent not on particles but on peptides and it is our analysis

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TABLE I. Stock Medium for *Glaucoma* (G) and *Colpidium* (C). Amounts in γ /ml of final medium.

	G	C		G	C
I. Amino acids			V. Vitamins		
DL-alanine	55	—	Ca pantothenate	1.0	1.0
L-arginine HCl	43	—	Nicotinamide	.1	.1
L-aspartic acid	61	—	Pyridoxal HCl	1.0	1.0
Glycine	5	—	Pyridoxamine HCl	1.0	1.0
L-glutamic acid	116	—	Riboflavin	.1	.1
L-histidine HCl \cdot H ₂ O	21	—	Thiamine HCl	1.0	1.0
DL-isoleucine	63	—	Folic acid	.01	.01
L-leucine	97	—	Biotin (free acid)	.005	.005
L-lysine HCl	76	—	Choline Cl	1.0	1.0
DL-methionine	34	—	DL-6-thioctic acid	.004	.004
L-phenylalanine	50	—			
L-proline	87	—	VI. Salts		
DL-serine	77	—	MgSO ₄ \cdot 7H ₂ O	100.0	100.0
DL-threonine	44	—	Fe(NH ₄) ₂ (SO ₄) ₂ \cdot 6H ₂ O	25.0	25.0
L-tryptophan	12	—	MnCl ₂ \cdot 4H ₂ O	.5	.5
DL-valine	66	—	ZnCl ₂	.05	.05
II. Protein			CaCl ₂ \cdot 2H ₂ O	50.0	50.0
Casein (Labeo)	10000	10000	CuCl ₂ \cdot 2H ₂ O	5.0	5.0
III. Carbon source			FeCl ₃ \cdot 6H ₂ O	1.25	1.25
Glucose	2500	2500	K ₂ HPO ₄	500.0	400.0
Tween 80	5000	10000	KH ₂ PO ₄	500.0	600.0
IV. Nucleic acid derivatives					
Guanylic acid	30	30			
Adenylic acid	20	20			
Cytidylic acid	25	25			
Uracil	10	10			

of the growth conditions for these 2 organisms which is to be reported here.

Materials and methods. *Glaucoma scintillans* A is the strain originally isolated and established in axenic culture in this laboratory (1). It was found that it could be adapted to a non-particulate medium (proteose-peptone, liver fraction L, glucose) by loop transfers, providing sufficient time was allowed for the initial incubation (10 days). That this was adaptation and not mutation and selection was shown by isolating single cells into a large number of separate tubes of the non-particulate medium, in which case all produced flourishing cultures after a lag of many days. After the cultures had reached high concentrations and small numbers were transferred to fresh non-particulate medium growth was rapid, reaching maximum yields in 96 hours. Early failure to achieve growth in non-particulate medium was due to insufficient time allowed for adaptation. Organisms once adapted to non-particulate medium can be subcultured

indefinitely and these were used for the experimental work.

The original strain of *Colpidium campylum* (1) has not been maintained. The strain reported here was obtained from the culture collection of Cambridge University, England. It was freed of associated bacteria and yeasts in this laboratory by a combined treatment with antibiotics and sulfonamides and a subsequent migration through small bore U-shaped tubing. This strain will be designated *Colpidium campylum* C.

The technics described previously in studies on *Tetrahymena* (3,4) were used here. All experimental setups were in triplicate, incubation tubes were slanted for maximum aeration and incubation times were 4 days for *Glaucoma* and 7 days for *Colpidium*. The initial pH of the media was 6.8 for *Glaucoma* and 6.0 for *Colpidium*. As was previously shown (1) the optimum pH for *Colpidium* is 5.4, but when using casein in the medium this much acidity results in turbid media, useless for turbidimetric measurement of growth.

Results. Early trials showed that neither ciliate would grow in the defined medium of *Tetrahymena*(5). The addition of proteose-peptone or casein to this medium did not improve it. It was found, however, that growth was limited by the level of pantothenate in the *Tetrahymena* medium and when this level was raised 10-fold, and casein added, good growth resulted. The pantothenate requirement will be discussed in a later communication.

Even with the high level of pantothenate neither organism grew in the absence of high molecular weight nitrogenous compounds. In the case of *Glaucoma*, the addition of peptone or casein hydrolysate (enzymatic) to a modified *Tetrahymena* medium (Table I) resulted in good growth, but not up to the level of whole casein. Casein was subjected to controlled digestion with a number of proteolytic enzymes but even short term action by the enzymes always reduced and never increased the activity.

To test the possibility of an unknown factor being a contaminant of the casein, thereby resulting in its activity, prolonged Soxhlet extraction with 95% ethanol was carried out. The casein was found to produce better growth after extraction than before (Table II), indicating that the growth-promoting activity resides in the casein and not in contaminating materials.

Bovine serum albumin (Armour) was substituted for the casein. The protein solution was dispensed aseptically after other components of the medium were sterilized by autoclaving. This protein was utilized readily

TABLE II. Response of *Glaucoma scintillans* A to Various Proteins and Protein Digests, Tested in "Stock Medium" (Table I) Minus II (Protein) and Plus Aspartic Acid (2 mg/ml). Figures represent optical density $\times 1000$.

Additions	Amount added (mg/ml)*		
	1	5	10
Labeo casein	214	479	585
" " ethanol extracted	235	556	623
Bovine serum albumin (Armour)	258	553	546
Casein enzymatic digest (NBC)	73	328	472
Proteose-peptone (Difco)	134	395	553
Tryptone (Difco)	155	340	434
Gelatin (Eastman)	63	121	132

* No growth with zero addition.

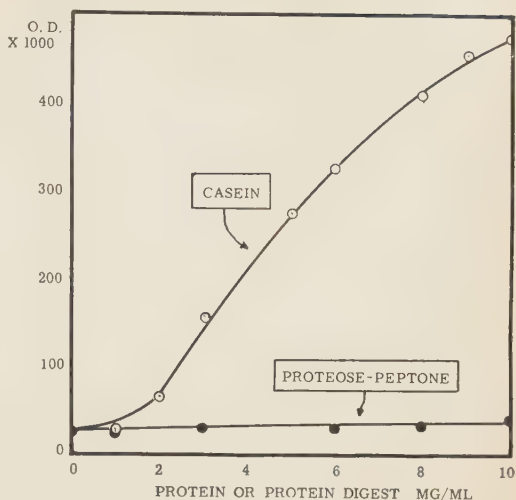


FIG. 1. Dose response of *Colpidium campyllum* C to Labeo casein and Difco proteose-peptone. The stock medium for *Colpidium* (Table I) minus protein was used at pH 6.0. Incubation time 7 days.

by *Glaucoma* (Table II) but not by *Colpidium*. Growth of *Colpidium* was obtained only when casein was present in the medium (Fig. 1).

Glaucoma can utilize free amino acids as evidenced by the fact that growth is increased by their addition (Fig. 2). The most active amino acid is aspartic acid. Asparagine is inert. With optimum casein (10 mg/ml) the addition of high levels of aspartic acid (2 mg/ml) increases the growth rate and raises the maximum yield. High levels of glutamic acid produce a similar effect, while the other amino acids (Table I) do not show marked stimulation individually but increase growth when added together (Fig. 2) even in the presence of high aspartic acid.

Free amino acids are inhibitory to *Colpidium*. The addition of single amino acids or combinations of amino acids to casein-containing media always reduces growth.

Discussion. The most striking difference between *Glaucoma* and *Colpidium*, on the one hand, and *Tetrahymena*, on the other, is the absolute requirement for polypeptides by the former two. *Tetrahymena* grows well on media based on crystalline amino acids(5). *Glaucoma*, while able to utilize free amino acids, is dependent on polypeptides. These do not seem to be specific, or if so they are not

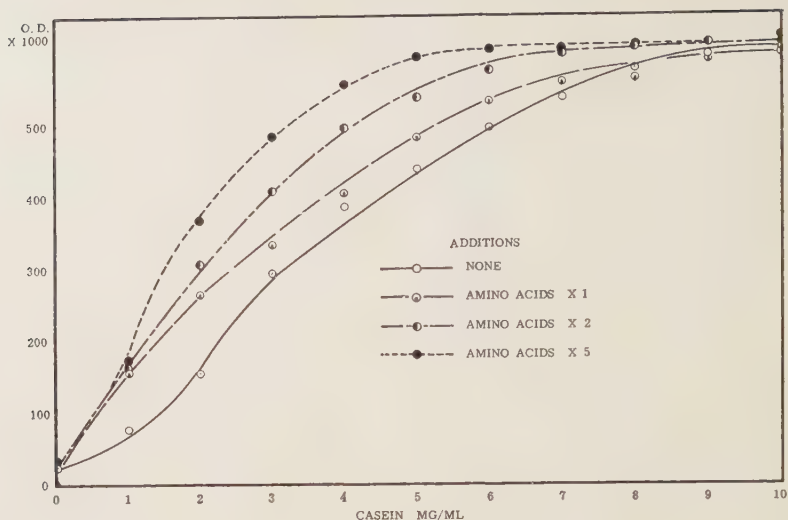


FIG. 2. Dose response of *Glaucoma scintillans* A to increments of Labeo casein. The medium is the stock medium (Table I) amino acids (I) minus protein (II) and plus aspartic acid (2 mg/ml). Amino acids \times = multiples of amino acids (I) in stock medium. Incubation time 96 hr.

released and made more available by various enzymatic reactions *in vitro*. Casein, treated to concentrate streptogenin(6,7) was used. In all trials the fractions containing streptogenin exhibited very low activity compared to the non-streptogenin containing fractions. The molecular size of the protein molecule appears to be the limiting factor in the growth of *Colpidium*. While the large casein molecule is adequate for this organism the much smaller albumin molecule is inert.

It seems possible that the basis for the above results lies in differences in reactions of the organisms regarding food-taking. While all 3 ciliates (*Tetrahymena*, *Glaucoma*, *Colpidium*) are bacteria feeders in nature, where the food organism evokes a swallowing response (food vacuole formation), only *Tetrahymena* has retained the ability to respond with vacuole formation to relatively small molecules in solution (drinking response). The swallowing response of *Glaucoma* is evoked by polypeptides and small proteins, while only large proteins evoke the response in *Colpidium* (Fig. 1). There are many ciliates in nature which appear to be much more exacting in their requirements for stimulation of the feeding mechanism than is *Colpidium* (e.g. *Bresslaui*(8), *Perispira*(9),

etc.)), where only certain types of organisms will be accepted as food.

When polypeptide and whole protein are required for growth it is extremely difficult to determine the synthetic capacities of organisms regarding specific amino acids. While we know nothing of these specific capacities in the case of *Colpidium* it has been possible to show with *Glaucoma*(10) that, under certain conditions and with very low protein additions (producing low but transplantable growth), the omission of any one of the following amino acids results in immediate growth failure: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, serine, and proline. These, with the exception of serine and proline, are the "essential" amino acids for *Tetrahymena* (11,12). The failure in an animal to synthesize proline seems very unusual, but under certain conditions even *Tetrahymena* fails to synthesize proline and also serine(13).

The growth factor requirements, aside from that for high levels of pantothenic acid, mentioned above, are similar to those of *Tetrahymena*(3). Some interesting differences, however, will be discussed later.

This investigation is important for practical purposes in isolating and establishing new or-

ganisms in axenic culture. Attention must be directed to both the kind and amount of biologically active molecules present, and especially to the complexity of the nitrogenous molecules. In these 2 cases, for instance, the amount of pantothenic acid in the *Tetrahymena* medium(5) plus that found in peptones is far below the amount required for growth. Yeast extract supplies the required pantothenic acid level but not the required polypeptide or protein level. For initial establishment in axenic culture, therefore, it is not always correct to assume that crude peptones or extracts of natural origin will supply adequate amounts of the compounds usually regarded as being essential.

The requirement for a polypeptide or protein is not unknown among organisms. Sprince and Kupferberg(14) have found that the flagellate, *Trichomonas vaginalis* can be grown on a pancreatic digest of casein to which are added the B-vitamins, purines, a pyrimidine, acetate, ribose, asparagine, linoleic acid, and serum albumin. While the essential nature of all the ingredients could not be determined, growth was dependent on the protein supplement.

Similarly, Dougherty *et al.*(15) have found that rhabditoid nematodes (*Rhabditis*), when grown in axenic culture, require a heat labile protein found in liver and chick embryo juice.

Recently Williams and Grady(16) have reported that a specific protein (lactein) is an absolute requirement for the growth of a strain of the bacterium, *Lactobacillus bulgaricus*. This requirement appears to be for a specific configuration, as in the case of streptogenin(6,7) rather than for a certain molecular size.

Summary. The nitrogenous requirements of *Glaucoma scintillans* and *Colpidium campylum* in axenic culture have been investigated. Both ciliates require approximately 20 times as much pantothenic acid as does *Tetrahymena pyriformis* W. *Colpidium* appears to require whole protein molecules of

the molecular weight range of casein. Digests of casein do not support growth, nor does bovine serum albumin. Free amino acids do not appear to be utilized and are somewhat inhibitory. *Glaucoma* will utilize free amino acids but will not grow when they are the sole nitrogen source. It appears to require peptides (as in enzymatic digests of casein or in peptones) but best growth occurs when whole protein molecules are present (casein or bovine albumin). The requirements of neither ciliate appear to be for a specific configuration (certainly not streptogenin) but rather for molecules of a certain minimum size. This may reflect the necessity for stimulation of the cell for the swallowing reaction.

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Effects of Growth, Fasting, and Trauma on the Concentrations of Connective Tissue Hexosamine and Water. (21203)

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Hexosamines* are present in the ground substance of connective tissue as components of mucopolysaccharides(1) and mucoproteins (2). Since the ground substance is altered in the collagen diseases, it has been suggested by many that changes in mucopolysaccharides may be related to these observed lesions. Schlamowitz *et al.*(3) have demonstrated that "nodules", experimentally induced by injections of trypsin and other enzymes, contain increased concentrations of hexosamine. This has also been noted in nodules obtained from patients with rheumatic fever(2) and in the corium and subcutaneous connective tissue of patients with scleroderma(4). In order to evaluate further the factors influencing connective tissue hexosamine levels, the effects of growth, fasting and various traumatic agents have been studied.

Methods. Male Sprague-Dawley rats were individually caged, fed a stock diet(5) and were permitted to drink water *ad libitum*. Rats of similar weight were used in each experiment. At the end of an experiment the rats were killed with ether. In those instances in which orbital connective tissue was obtained the rats were killed by exsanguination under ether anesthesia. Subcutaneous connective tissue samples were obtained from an area of the back, caudal to the scapular adipose tissue. The skin was cut along the midline of the back, retracted and the sample (50-200 mg) removed by scraping the loose connective tissue from the underside of the corium with a pair of wing-tipped forceps. Orbital connective tissue was obtained following removal of the eyeballs by dissection from the Harderian glands. After weighing, all the samples were placed in an oven at 101-104°C

for 18 hours, reweighed, then extracted in a Soxhlet for 3 hours with redistilled petroleum ether (B.P. 60-70°C). The dry, fat-free samples were weighed and the water and fat contents calculated. The amount of fat in a wet sample of subcutaneous connective tissue may vary from 2-40%, causing a wide variation in calculated water and hexosamine values from sample to sample. The concentrations of tissue hexosamine and water are independent of the amount of fat present, therefore, all expressions of concentration for water and hexosamine are presented relative to fat-free tissue. Water is expressed as per cent (wet wt—dry wt) $\times 100/(\text{wet wt} - \text{fat})$, and hexosamine as mg/100 g of fat-free dry tissue. To determine the hexosamine content, the dry defatted samples were each placed in 10-ml glass-stoppered volumetric flasks. After adding one ml of 4 N hydrochloric acid, they were hydrolyzed 15 hours at 100°C, taken to volume with water, and filtered. Hexosamines were determined by a modification of the Elson and Morgan method after separation from the hydrolysates on columns of Dowex-50(6).

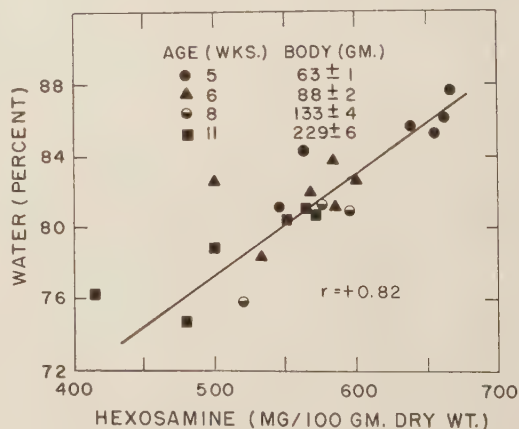


FIG. 1. Correlation between water and hexosamine concentrations in subcutaneous connective tissue of rats of varying ages.

* The 2 amino sugars normally encountered in connective tissue are galactosamine and glucosamine. The method of Elson and Morgan used here does not distinguish between them.

Results. Growth. Four groups of rats, 5, 6, 8, and 11 weeks of age, with 4-6 rats in each group, were used. Subcutaneous connective tissue samples were obtained and analyzed (Fig. 1). The water and hexosamine concentrations, high in the youngest group, decreased with increasing age. Water and hexosamine values obtained on the same samples in each animal were found to have a high degree of correlation ($r = +0.82$), which was significant at a probability level of <0.01 .

The effect of body weight on the concentrations of water and hexosamine in orbital connective tissue was similarly studied by analyzing the data from 93 rats which had served as controls in several different experiments. Correlation studies were made between body weight (range was 60-235 g) and hexosamine concentration (range was 357-493 mg/100 g dry wt), body weight and water concentration (range was 76.6-79.8%), and hexosamine and water concentrations. There was a significant negative correlation between hexosamine concentration and body weight ($r = -.81$), and also between water concentration and body weight ($r = -.77$). Thus, with increasing body size there was a progressive decrease in the concentrations of hexosamine and water. The hexosamine and water changes were tested and found to have a significant positive correlation ($r = +.69$). In each instance the values for r were significant at a probability level of $<.01$.

Fasting. A reduced food intake decreases the amount of plasma hexosamine(5). It was therefore considered of interest to assess the effect of fasting on the concentrations of hexosamine in normal connective tissue. Sixteen rats were used, and sacrificed in groups of 4. One group was killed at the start of the experiment and the remaining groups following one, 2, and 3 days of total fasting. The rats had drinking water available. Fasting had no significant effect on the concentrations of hexosamine which were 581 ± 20 , 599 ± 20 , 577 ± 6 , and 584 ± 11 mg/100 g dry wt on 0, 1, 2, and 3 days, respectively. There was a decrease in connective tissue water, most striking on the second day ($77.0 \pm .8\%$ as compared with $81.6 \pm .2\%$ at the start of

the experiment). The normal relationship between water and hexosamine was thus altered by the second day of fasting (compare with Fig. 1). During this fast the mean body weight fell from 109 ± 7 g at the start of the experiment to 80 ± 4 g on the third day. Since fasting did not affect hexosamine levels in connective tissue, food intake was not measured in subsequent experiments.

Local trauma. Following a preliminary observation that epinephrine injections increased the hexosamine concentration at an injection site, an experiment was designed to determine the rate of this change. In the first experiment, 45 rats weighing 110-130 g were used; 5 were used as an initial control group; a group of 20 rats was injected subcutaneously twice daily in the same site (right dorsum) with 0.1 ml of physiological saline; a group of 20 rats was injected similarly with 0.1 ml of epinephrine (1:3000). Five rats in each injected group were sacrificed at 6, 24, 48, and 72 hours and connective tissue samples obtained (Fig. 2). In both groups the concentrations of water and hexosamine increased, reaching highest observed values at 24 hours (epinephrine) and 48 hours (saline). The increases in the hexosamine levels in response to saline and epinephrine were not significantly different. At 24 hours, the increase in water concentration produced by epinephrine was greater than that produced by saline. In subsequent studies, a response time of 48

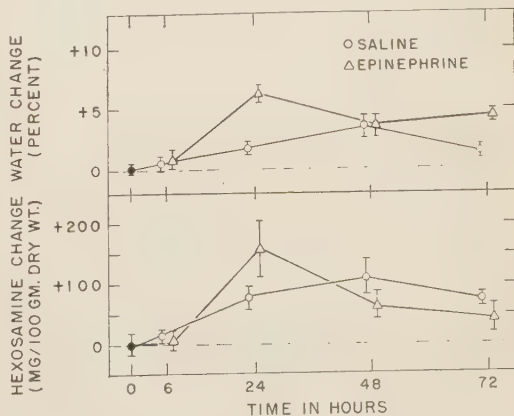


FIG. 2. Effect of saline and epinephrine injections on concentrations of hexosamine and water in subcutaneous connective tissue. Vertical lines refer to S. E. of mean.

TABLE I. Effect of Local Traumatizing Agents on Concentrations of Connective Tissue Water and Hexosamine.*

Traumatizing agent	No. rats	Body wt (g)	Water (%)	Hexosamine (mg/100 g dry wt)
Control	5	122 \pm 5	81.8 \pm 1.0	561 \pm 12
Formalin (0.25 ml of 2%)	4	112 \pm 4	89.6 \pm .4	580 \pm 16
Ammonia (0.25 ml of 1%)	5	117 \pm 6	81.4 \pm 1.3	601 \pm 27
Ethyl alcohol (0.25 ml of 50%)	4	120 \pm 6	87.3 \pm .6	616 \pm 26
Plier pinch	4	110 \pm 2	84.1 \pm .7	632 \pm 28
Incision	4	109 \pm 2	85.0 \pm .6	640 \pm 12
Venice turpentine (0.3 ml)	4	104 \pm 1	84.4 \pm .3	666 \pm 23
Tannic acid (0.25 ml of 4%)	4	111 \pm 2	85.0 \pm .4	736 \pm 26

* Mean \pm S. E.

hours was used for testing other noxious agents.

In the next experiment several traumatizing agents were tested for their effect on connective tissue hexosamine levels. All rats received a single stressing stimulus to the subcutaneous connective tissue of the lower right dorsum. Various groups received subcutaneous injections of either formalin, ammonia, ethyl alcohol, turpentine, or tannic acid. In one group the skin was squeezed firmly with a pair of pliers. In another group the skin of each animal was incised (2-3 cm) and sewn with silk sutures. The latter 2 injuries were produced under ether anesthesia. Samples were obtained 48 hours following the tissue trauma (Table I). In almost all instances the concentrations of water and hexosamine increased, but in varying degrees and not necessarily paralleling one another. Following formalin, for instance, there was a marked edema (89.6% water) with only minimal hexosamine changes (580 mg/100 g dry wt), whereas with tannic acid the edema was less pronounced (85%) but the hexosamine strikingly increased (736 mg/100 g dry wt). The response was generally characteristic for each agent. For example, repeated experiments demonstrated that an incision or tannic acid injection consistently induced significant increases in hexosamine concentration, whereas formalin usually failed to stimulate such a response despite its edema-inducing properties.

Fasting and local trauma. Since fasting prevents the expected increase in the plasma hexosamine level following body injury(5), the effect of fasting on the response of connective tissue to local trauma was studied. Three

groups of rats were used, 1) a control group eating *ad libitum*, 2) a group injected with tannic acid (.25 ml of 4% solution) eating *ad libitum*, and 3) a group injected with tannic acid after 24 hours of a 72-hour fast. All rats were sacrificed 48 hours following the time of the injection. The fasted group weighed 92 \pm 2 g, whereas the injected group eating *ad libitum* weighed 140 \pm 4 g. Although the fasted group had a reduction in water content (81.5 \pm 1.1% as compared with 85.3 \pm .8 in the injected group eating *ad libitum*), the hexosamine concentration increased the same amount in both injected groups (761 \pm 22 mg/100 g dry wt in the injected group eating *ad libitum* and 776 \pm 21 in the injected group which was fasted) as compared with the controls (561 \pm 10 mg/100 g dry wt).

Systemic stress. Since many types of body injury will produce a rise in the plasma hexosamine level(4), a systemic stress was induced to determine whether or not the hexosamine in non-traumatized connective tissue participates in this response. As a stress the right femur and tibia were fractured under ether anesthesia. Connective tissue samples from the dorsum were obtained from a control group at the start of the experiment and from groups 2, 4, and 8 days following the day of fracture. No significant changes were noted in the concentrations of hexosamine and water.

Discussion. The concentrations of connective tissue hexosamine and water decrease with growth. Similar observations on age changes have been made in the skin of the rat(7), where the decrease in the concentration of hexosamine was shown to result primarily from a relative increase in collagen. It is

likely that mucopolysaccharides, which account for approximately 50% of the total connective tissue hexosamine(8) and behave as hydrophilic colloids, would largely account for the parallelism between the tissue water and hexosamine concentrations. The close correlation between hexosamine and water has been observed in experimental myxedema(9), and in human skin(4). This relationship is altered in tissue from fasted rats and in traumatized tissue.

It is well recognized that following many types of body injury the plasma hexosamine level increases(3,5,10). The site of production or source of this hexosamine has not been defined. The synthesis of hexosamine by isolated connective tissue cells has recently been demonstrated(11) and it therefore is possible that the increased amount of plasma hexosamine following trauma may be produced by connective tissue. Although it was not possible to detect changes in hexosamine concentration in non-traumatized connective tissue following trypsin injections(3) or fracture, as reported here, the possibility that a concurrent increase in plasma hexosamine(3,5) may result from its synthesis by connective tissue is not excluded.

Summary. 1. The concentrations of hexosamine and water in connective tissue decrease with growth in the rat. There is a high degree of correlation between the concentrations of water and hexosamine in normal connective

tissue. 2. Most traumatic agents (physical and chemical) effect a local non-specific increase in hexosamine and water concentrations. The degree of change of each of these constituents varies with the agent administered. Fasting does not influence this increase in hexosamine in response to local trauma, or the hexosamine concentration in untraumatized connective tissue. 3. Changes in the concentration of connective tissue hexosamine were not detectable following systemic stress (fracture).

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Pantothenic Acid Deficiency Induced in Human Subjects.* (21204)

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Shortly after the classic demonstration by Woods(1) of the molecular antagonism of sulfanilamide by para-amino benzoic acid, one of us (WBB) became interested in seeking similar relationships between individual B-

complex vitamins and their chemical analogues. A series of pyridine compounds was studied for vasodilating effects and therapeutic potency against pellagra(2). Observations at that time suggested that some of the compounds might have toxic properties. This suspicion had been expressed by Woolley(3) as a result of his work with canine 'blacktongue'. McIlwain(4) suggested that pyridine-3-sul-

* Supported by the Medical Department Trust Fund and, grants from Eli Lilly Company, The Nutrition Foundation and the National Vitamin Foundation.

phonic acid, which antagonized the stimulating effect of nicotinic acid on bacterial growth, might hasten the onset or aggravate the severity of manifestations of pellagra in patients whose poor diets predisposed them to recurrent attacks each spring. Since nicotinic acid, a potent therapeutic agent, was available, an effort was started in 1941 to induce pellagra in patients at the Nutrition Clinic in Birmingham, Ala. In addition to a diet low in B-complex vitamins, these subjects were given injections intravenously of pyridine-3-sulphonic acid for 14 days. Their clinical condition did not change(5). The studies were interrupted for 6 years by the war. However, during these years the first effective use of a vitamin antagonist was reported for the newly discovered folic acid(6,7). Subsequent studies by Vilter and his colleagues(8) demonstrated the feasibility of inducing specific vitamin deficiency states by means of other antagonists. This field of investigation, still in its infancy, has already yielded results in therapy of the leukemias, some neoplastic diseases and in thromboembolic conditions.

The pathfinding studies of Williams(9) in discovering pantothenic acid and of Lipmann(10) in elucidating some of its many functions, have been reviewed elsewhere. Studies of pantothenic acid deficiency in animals have indicated that the principal manifestations are those of adrenal cortical insufficiency, accompanied by low levels of plasma cholesterol, gastrointestinal symptoms, neurological lesions, faulty antibody production and epidermal changes(11-13). Since the role of pantothenic acid in human economy is known only by inference, we undertook a study of this deficiency in healthy young volunteers. With the principle of metabolic antagonism well established, and with injectable pantothenic acid available, we employed antagonists despite some recognized potential hazards(14). Two problems delayed progress. First, we found that most of the foods contained more pantothenic acid than was estimated from the tables, so we finally resorted to a purified and chiefly synthetic diet which was supplemented by known vitamins exclusive of pantothenic acid. This diet alone resulted in some minor biochemical alterations, such as

lowering of the plasma cholesterol concentration, and impairment of the eosinopenic response to ACTH, but clinical signs of deficiency did not appear. We next tried the diet plus an antagonist, pantoyltaurine, increasing the dose by small increments until we found that it was inert in amounts many hundreds of times greater than the average daily pantothenic acid intake. The opportunities for testing various clinical and biochemical events seemed almost without limit. We chose those which reflected cortical activity, hepatic function, and acetylation processes. These studies, conducted over 4 years on the Metabolic Ward and supported by generous grants from pharmaceutical manufacturers, various foundations, government agencies, and departmental earnings, set a pattern for our present successful study of human pantothenic acid deficiency.

Methods. We first induced a deficiency in a single subject who was given the deficient diet plus a new antagonist, omega methyl pantothenic acid. From this pilot study we formulated an experimental design which was applied to 3 additional subjects simultaneously. The subjects were 4 healthy men ranging in age from 19 to 31 years. One had bronchial asthma, but was otherwise well. The other 3 were without illness. The plan was to make baseline studies for a 12-day period. During this time the men were to receive a 'basic diet' fully supplemented by all nutritional requirements. The second period was to be identical except for elimination of pantothenic acid from the diet, and substitution of 0.5 g of omega methyl pantothenic acid daily. Its duration was to depend upon clinical or biochemical evidences of a deficiency. Finally, we planned a third period, identical with the second, but adding large amounts (4 g) of pantothenic acid to overbalance the effects of the antagonist. We proceeded according to this schedule, through the first period, and the second which was ended when certain abnormalities developed. However early in the third period, when it became evident that the clinical manifestations of the deficiency were progressing despite the addition of pantothenic acid, we promptly abandoned the proposed schedule and instituted emergency therapy

TABLE I. Clinical and Laboratory Changes in 3 Men Deficient in Pantothenic Acid.

	I	II	III	IIIa	IIIb
	Control 12 days	Deficiency 35 days	Replacement 6 days	Emergency therapy 10 days	Recovery 14 days
	Basic diet Vitamins Minerals	Basic diet Vit. (-P.A.) Minerals Antagonist	Basic diet Vit. (+P.A.) Minerals Antagonist	Cortisone (3d.) General diet Parenteral B-vit. and P.A.	Basic diet Vitamins Minerals
Clinical observations					
Wt	N	N	N	++ (edema)	N
Blood pressure, syst./diast.	N/N	±/—	±/—	+/+	N/N
Skin & mucous membranes	N	N	N	N	N
Personality	N	—	—	+	N
Gastro-intestinal changes	N	++	++	+	N
Appetite	N	—	—	+	N
Neurologic stepp. gait	0	+	+	0	0
paresthesias	0	+++	0	0	0
tendon reflexes	N	++	++	N	N
vertigo	0	++	+++	0	0
burning feet	0	+	+	0	0
Sense of well being	N	—	—	±	N
Laboratory tests					
17 ketosteroid	N	—	—	—	N
Eosinopenia—ACTH	+	—	±	+	+
Cholesterol and esters	N	—	—	—	—
Blood counts—RBC	N	N	N	N	N
Hb	N	N	N	N	N
Wbc	N	N	N	N	N
Eos	N	+	+	N	N
Diff.	N	N	N	N	N
Urinalysis—Sugar	0	±	±	±	0
Albumin	0	0	Tr	Tr	0
Micro	N	N	N	N	N
Urobilinogen	Tr	0-+++	0-+++	0-+++	Tr
Glucose tolerance	N	±	±	±	N
Insulin "	N	±	—	±	N
Water diuresis	N	—	—	±	N
Gastric acidity	N	—	—	±	N
Plasma proteins	N	N	N	N	N
BSP, 5 mg/kg/45 min.	N	N	N	N	N

Key: N = Normal, + = Increase, — = Decrease, 0 = Absent, Tr = Trace.

until recovery was clinically evident. At this time the third period was resumed, and observations again obtained. Among the measurements selected to detect early signs of deficiency, we included daily recordings of body weight, blood pressure, urine volume, acetylation of administered PABA, and excretion of 17 ketosteroids. Twice each week, we determined plasma cholesterol and cholesterol esters, eosinophil response to ACTH, and gastric acidity. Once a week, we obtained a complete blood count and urinalysis. The glucose tolerance, insulin tolerance, and response to water diuresis were also observed weekly. Each 14 days we took a photograph of the men and determined plasma proteins,

plasma electrophoretic patterns and brom-sulfalein extractions.

Results. Clinically, no changes were evident during the first period. But in the second week of the period of deficiency we noted a fall in the diastolic and lability of systolic blood pressure. Postural hypotension and vertigo developed, accompanied by tachycardia after slight exertion. The men complained of easy fatigability and slept frequently during the daytime. By the third week of the deficient period, they complained of occasional bouts of epigastric distress accompanied by anorexia and constipation. One subject regurgitated occasionally, necessitating caloric replacements. The fourth week found

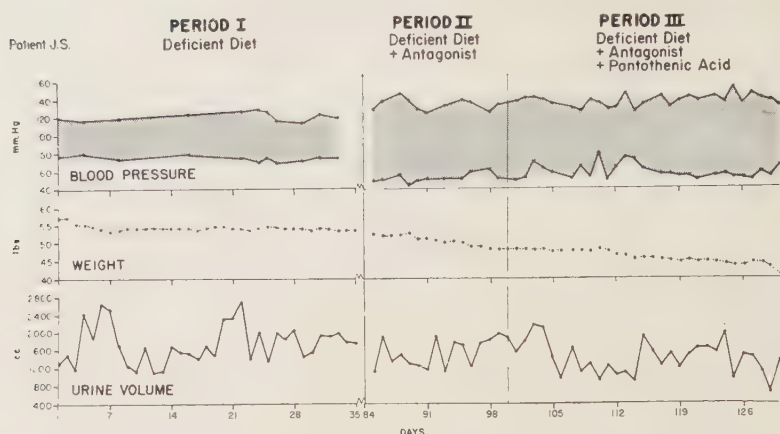


FIG. 1. Pt. J.S. Note development of wide pulse pressure accompanied by progressive wt loss despite dietary intake of 3000 calories with 100 g of protein daily. Addition of pantothenic acid did not promptly reverse these changes.

the subjects discontented, quarrelsome, irascible, and easily upset. They complained of increasing sensations of numbness and tingling of the hands and feet. In the following week, these sensations became more annoying, and in one man consisted of a constant burning of the feet. Another developed a steppage gait, while the third complained of constant paresthesias. Neurological examination disclosed hyperactive deep tendon reflexes, inability to walk on tip-toe, weakness of the extensor muscles of the fingers, and impaired sense of balance. Nevertheless, objective sensory examinations were normal as were the plantar reflexes. During this entire period of deficiency, the men had frequent upper respiratory infections, especially acute pharyngitis, which previously had been infrequent. One developed pneumonia which responded rapidly to antibiotic therapy.

At this time, it was deemed necessary to restore pantothenic acid to their diets, so the third period was started. Although the paresthesias improved promptly, and the eosinopenic response to ACTH was partially restored, the patients became more fatigued, and their sense of well-being deteriorated. At the same time, their urinary excretion of 17 ketosteroids became lower. Severe vomiting suddenly developed in one patient, while another became somnolent and lethargic for an entire day, although he had no further alterations in his pulse, respirations, blood pressure or tem-

perature. These alarming events, reminiscent of acute adrenal insufficiency, led us to institute prompt therapy with fluids intravenously and cortisone parenterally in the first patient, and cortisone orally in the other 2. They were also given a general diet supplemented by multiple vitamins and injections of B-complex vitamins and pantothenic acid. In response to this therapy, the men improved rapidly and cortisone was discontinued after 3 days. The diet and vitamins were continued for another 7 days during which time the men gained weight rapidly (10 to 13 lb), and developed edema and elevated blood pressure. At the same time, their urinary excretion of 17 ketosteroids declined still further. The next 2 weeks, during which time the "basic diet" supplemented by vitamins was resumed, were characterized by gradual recovery both clinically and biochemically. No further evidence of neuropathies occurred, but one man who had had thrombophlebitis 8 years previously, had a mild recurrence with residual edema of the affected foot and leg.

The general features of biochemical changes accompanying pantothenic acid deficiency, presented in Table I, are characterized by impaired ability to acetylate PABA, and a decline in blood levels of cholesterol and cholesterol esters. Some evidences of adrenal cortical hypofunction are found in the eosinophil response to ACTH, the increased sensitivity to insulin, the decreased urinary excretion

17-KETOSTEROID EXCRETION

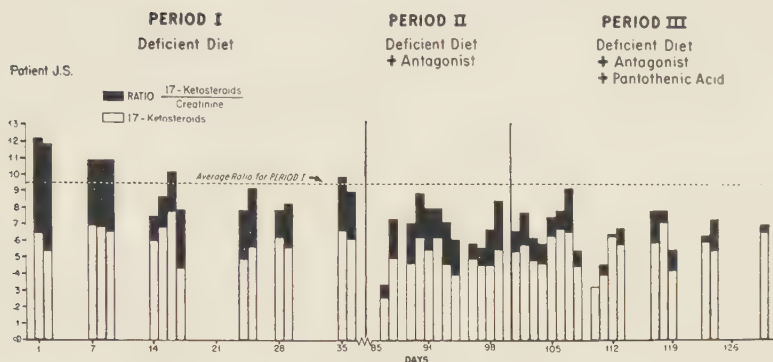


FIG. 2. Pt. J.S. Urinary 17 ketosteroid excretion.

of 17 ketosteroids and the defective diuresis after water ingestion. Similar events occurred in our first subject (Fig. 1, 2, and 3). One unexpected finding was gastric hypochlorhydria, and in one subject, histamine fast achlorhydria. These changes returned to normal during the recovery period. Many of our data are yet to be assembled and evaluated critically. The details of these observations will be the subject of a later report.

Discussion. From these studies, it is apparent that pantothenic acid, or its metabolic derivative, coenzyme A, is essential to the human economy. The spontaneous develop-

ment of such a deficiency is unlikely since this vitamin is so widely distributed in foodstuffs, and is remarkably resistant to destruction by thermal or chemical agents. Development of an abnormal state by such artificial means is justified by the information derived therefrom. Apparently acetylation processes are essential to the integral function of the adrenal cortex, either in supplying cholesterol to this gland as a substrate, or in conversion of the substrate into steroid hormones, or both. What role acetylation plays in gastric secretion of hydrochloric acid is not apparent, but perhaps this is related to the functional level of the adrenal

EOSINOPENIC RESPONSE TO ACTH

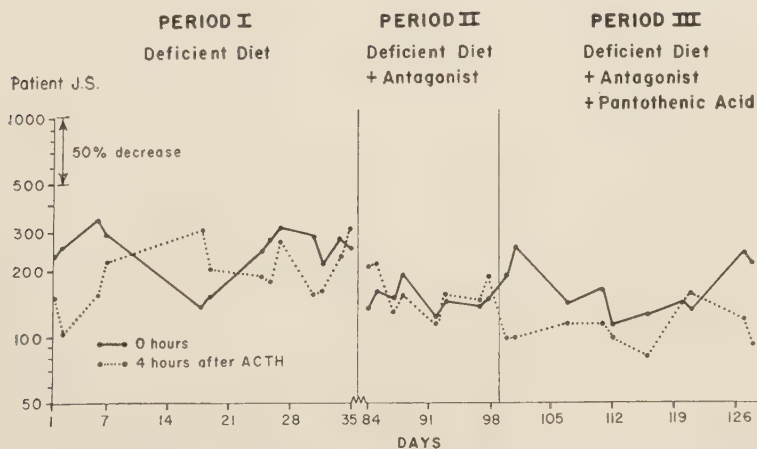


FIG. 3. Pt. J.S. Eosinopenic responses to ACTH were somewhat variable, but disappeared entirely during period of deficiency and returned partially when pantothenic acid was restored to the diet. Note that by using a semilogarithmic graph the distance between points remains constant for any given per cent of eosinopenic response regardless of initial count.

cortex since adrenal insufficiency is frequently accompanied by achlorhydria, and therapy with ACTH is accompanied by hyperchlorhydria. Finally, the development of peripheral neuropathy is similar to that in swine described by Wintrobe.

Summary. 1. An abnormal metabolic state has been induced in 4 human volunteers through combined use of a diet deficient in pantothenic acid and a metabolic antagonist, (omega-methyl pantothenic acid). 2. This abnormal state was accompanied by clinical and biochemical abnormalities suggesting adrenal cortical insufficiency, and by a peripheral neuropathy. 3. Administration of pantothenic acid alone did not immediately reverse the abnormal state. A good diet of natural foods and multiple vitamins resulted in rapid complete recovery.

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Sensitivity of Females of the C Stock to Male Infection with the Mammary Tumor Agent.* (21205)

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The mammary tumor agent (MTA), one of the factors usually required for the development of a high incidence of spontaneous mammary cancer in mice(8,9), may be administered by the injection of extracts of either normal or cancerous tissues from infected animals(10), including seminal vesicles(4) and cauda epididymis(15). Following the mating of agent-free females of some strains, which are either susceptible or resistant to the

development of this type of cancer, with males of cancerous stocks, the appearance of mammary cancer in either the females or their progeny has been explained as resulting from the transmission of the MTA by the male at the time of coitus(3,7,11-17). Differences have been found in the sensitivity of agent-free females of various strains to become "infected," as well as the ability of males possessing the agent to infect females of either the same, or other stocks(12).

Females of the C (Bagg albino) stock have been found to be susceptible to the development of mammary cancer, as determined by a high incidence in mice which possessed the MTA, obtained by nursing females of a cancerous strain(1).

This report considers the sensitivity of

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agent-free females of the C stock to become infected with the agent after being mated with males of different cancerous stocks, as determined by the incidence of mammary cancer in the C females. As controls agent-free males were tested with C females in 2 studies.

Materials and methods. Males of the cancerous A, C₃H, and dilute brown stocks, possessing the MTA, were used in the present investigations. Males of the Andervont(1-7, 15) subline of the C₃H stock, maintained in this laboratory since 1948, will be referred to as the C₃H/An line, while our subline has been called the Z(C₃H) line(10-12). Other Z males were used which were descended from females which had been nursed on a female of the cancerous A stock. These will be called Za (Z with the agent from the A stock) males, to distinguish them from the typical Z animals. The Za males were of the second generation, since fostering. The Zb males were from the fostered subline of the Z stock, and were free of the MTA. The mice of the cancerous dilute brown stock were of the D₈ subline(10). In most instances, when females of the C strain were mated with males of other strains, 5 females were mated with each male, and they were permitted to have as many litters as they would before they either developed cancer or died of other causes. The females were usually separated from the males, except in force breeding experiments, before their litters were born. The mice received Purina fox chow and tap water.

Results and discussion. The incidences of mammary cancer, with the average cancer ages given in days, for females of the different inbred stocks are recorded in Table I. The data are for mice observed during the same periods as represented in Table II, except for the control C stock.

The observations for the C stock were for breeding females born between 1943 and 1951, and included only the mice which were continued for at least 300 days. Of the total, 71% lived for more than 450 days, and 35% were maintained for longer than 600 days; the average ages at death for mice of the 2 later groups were 593 and 720 days, respectively. Of the C females which survived for longer than 600 days, all had at least 5 litters,

TABLE I. Observations on Development of Mammary Cancer in Inbred Strains Maintained during Period of the Experimental Studies.

Stock	Data for breeding females			Data for virgin females		
	No.	Cancer (%)	Ca. age	No.	Cancer (%)	Ca. age
A	352	90	302	—	—	—
Z(C ₃ H)	589	97	273	37	86	399
Za	75	92	316	27	67	390
C ₃ H/An	127	98	229	117	94	354
D ₈	150	76	458	—	—	—
C	252	0	—	—	—	—

82% had 7 or more litters, and 28% had at least 9 litters each. All C females which had been mated with their brothers, to continue the inbred stock, died without mammary cancer.

Virgin females of the A strain were not observed during the course of the present study, but the incidence of mammary cancer was determined at previous periods and found to be no higher than 5% (8-10). Several of the females of the Za group are still living and the data for mammary cancer are for mice of litters in which all animals are now dead. While the same percentage of the breeding females of the D₈ stock developed mammary cancer as reported previously(10), the cancer age for the present series was later (405 vs. 458 days, respectively). The D₈ virgins in the group observed earlier showed an incidence of 66%, with an average age of 459 days.

The C females which were mated with males of other stocks are tabulated in Table II, by experiments. Mice of Groups 2 and 6, and 4 and 5 were litter mates, separated so that approximately the same number from each litter was placed in each series. The females of Group 2 were maintained as force breeders, and those of Groups 4 and 6 were force bred part of the time. Other females (Group 5) had their uterine horns amputated at the junction with the body of the uterus, leaving the ovaries *in situ* and intact. These females were housed with the same males as their intact litter-mate controls (Group 4). The C females mated with males of the Z(C₃H) stock were born between 1947 and 1951; the others between 1949 and 1951.

TABLE II. Incidence of Mammary Cancer in Females of the C Stock when Mated with Males of Other Stocks.

Group	Stock of males	No.	Cancer (%)	Avg age in days		Avg No. litters		Females born
				Cancer	Nonca.	Cancer	Nonca.	
1	Z(C ₃ H)	8	50	544	686	9.0	10.3	1947
2	Z(C ₃ H)	14	64	483	688	9.9	9.6	1949
3	Z(C ₃ H)	5	60	558	533	8.0	7.5	1950
4	Z(C ₃ H)	14	50	495	720	8.0	6.5	1951
5	Z(C ₃ H)	17	53	551	666	—	—	1951
1-5	Z(C ₃ H)	58	55	520	678			
6	A	15	33	554	599	10.4	9.2	1949
7	C ₃ H/An	10	10	636	616	8.0	7.0	1950
8	C ₃ H/An	8	25	684	597	9.5	6.0	1951
9	D ₈	10	0	—	676	—	8.3	1951
10	Za	9	22	482	646	8.5	9.0	1952
11	Zb	14	0	—	671	—	8.4	1949 & 51

Five to 17 C females, in 5 separate studies, were mated with males of the cancerous Z(C₃H) stock, and in each series from 50% to 64% developed mammary cancer (Table II). The average cancer age for the total was 520 days, and the cancerous females average from 8 to 10 litters. Whereas Muhlbock(16) did not observe the development of mammary cancer in sterilized females of 2 groups of hybrids which had been mated with males possessing the MTA, in the present study the same incidence was found in those with amputated uterine horns (Group 5) as in unoperated, litter-mate controls (Group 4).

When Z males possessing the agent from the A stock were tested, 2 of the 9 C females mated with these Za males had mammary cancer (Group 10). The noncancerous females lived to an average age in excess of 21 months and cast an average number of litters equal to those which showed higher incidences of cancer.

Fifteen females of the C stock were mated with males of the cancerous A strain (Group 6), 18 with C₃H/An males (Groups 7 and 8), and 10 with D₈ males (Group 9); the respective incidences of mammary tumors were 33%, 17%, and 0%.

Two series of C females, totaling 14 mice, were housed with agent-free males of the Zb subline and all died without mammary cancer, after giving birth to an average of 8.4 litters (Group 11, Table II). This incidence was the same as that noted in the control C females (Table I).

In previous reports(11-12), data were pre-

sented on the development of mammary cancer in the hybrids derived by mating females of the C stock with males of the cancerous Z(C₃H) stock. These observations have been corroborated in the present series. Also, by observing the incidence and age of development of tumors in the hybrids, the time of infection of the females by the males could be ascertained. The few mammary tumors which appeared in the progeny prior to this time occurred in old mice, but after the C mothers became infected, the incidence in their CZF₁ progeny was in excess of 90% and the average cancer age was approximately 8 months. The data indicate that the C females might become infected between the birth of their third and eighth litters, and that the females need not develop mammary cancer to become infected with the MTA by males of the cancerous stock. Several mammary tumors, from C females and their CZF₁ progeny, were tested by biological assay and found to carry the MTA.

To obtain some material on the time relationship between the administration of the MTA and when it might be transferred in the milk, C females which were 4 to 6 months of age were injected with extracts containing the agent. The progeny born in their first litters showed a high incidence of mammary cancer. Other preliminary data demonstrate that if C females become infected with the agent from males of the cancerous Z stock, their progeny born in later litters but sired by agent-free Zb males continued to have a high incidence of mammary cancer.

Only preliminary data may be presented for the hybrids obtained from the other crosses, since many of them are still under observation, with the youngest animals being approximately 20 months of age.

Confirming the observations of Andervont and Dunn(1-4,6,7), in hybrids obtained by mating C females with the C₃H/An males more mammary tumors are being found among mice born in early litters than was seen in any other cross. Of those which have died, 40% have had mammary cancer at an average age of 580 days. When Za males were tested, the hybrids cast by C females after they became infected have shown an incidence of 86% at an average age of 275 days, with 6 of the 49 mice still living. No evidence that the D₈ males infected any of the C females was obtained. Of their 126 CD₈F₁ progeny which have died, 4% have had tumors at an average of 538 days. This compares with an incidence of 3% at 572 days in 102 CZbF₁ hybrids, all of which are now dead.

Summary. Females of the agent-free, but susceptible, C (Bagg albino) stock remained free of mammary cancer after they were bred with males of the C strain and with males of another stock lacking the mammary tumor agent (MTA). However, when they were mated with males of high cancerous strains, differences were noted in the ability of the males of these strains to transfer the agent

and infect the C females. After C females were mated with males of the cancerous Z(C₃H) strain, over 50% of the females developed mammary cancer, and the same incidence was observed in females with amputated uterine horns as in intact litter-mate controls. The source of the agent may be of some significance in male transmission experiments. In some crosses, only preliminary data could be reported in the hybrids.

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Effects of Ethionine upon Hepatic Glycogen Formation from Glucose in Intact Rats.* (21206)

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Ethionine (S-ethyl homocysteine), an analogue of methionine, has been found to induce a fatty liver in fasting female rats(1,2). Male

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rats and females pretreated with testosterone fail to develop this fatty change in the liver (3). Ethionine also inhibits, in female rats, the incorporation of labeled DL-methionine and glycine into liver protein *in vivo*(4) as well as the incorporation of L-cystine, DL-leucine, L-valine, and L-lysine into liver protein and plasma protein(5). This inhibition

occurs at least several hours before there is a detectable increase in liver lipids. Male rats fail to show this inhibitory effect on amino acid incorporation(5,6) but do share with females other inhibitory effects of ethionine on liver metabolism, such as inhibition of transmethylation from methionine to choline and inhibition of the conversion of the methionine methyl group to formate(6,7). Thus, a correlation has been found between the inhibition of amino acid incorporation into liver protein and the subsequent development of fatty liver in rats given ethionine.

In an effort to investigate further the pathogenesis of the fatty liver and to establish whether the correspondence between disturbances in protein and lipid metabolism is significant, other metabolic activities in the liver are being studied in animals which are either susceptible or resistant to the fatty change in the liver. This report is concerned with the influence of ethionine upon the conversion of glucose into hepatic glycogen.

Experimental. White Wistar rats (Carruth), weighing 150 to 250 g were fasted for 24 hours. Three series of experiments were performed: Series I—groups of male and female rats received by stomach tube 2.5 g of glucose, dissolved in 3 ml of water, at the end of the initial 24-hour fast period. These animals also received ethionine (experimental), an equal volume of saline (control), methionine (control), or a mixture of ethionine and methionine (experimental) by intraperitoneal injection. The ethionine group was given 0.75 mg DL-ethionine per g of body weight in 2 divided doses: one-half initially at the end of the 24-hour fast period and one-half 2 hours later. Methionine (DL) when used was administered in amounts equimolar to ethionine. Methionine and ethionine were dissolved in the same solution when given together. The rats were fasted until sacrificed 6 hours after the administration of glucose. Series II—groups of male and female rats received subcutaneously in 4 divided doses 1.3 g of glucose dissolved in 13 ml of water: 4 ml at the end of the initial 24-hour fast period, and 3 ml every hour thereafter for 3 hours. These animals also were injected with ethionine, methionine, ethionine plus methio-

nine or saline in the same manner as in Series I. The rats were fasted until sacrificed 6 hours after the first injection of glucose. Series III—groups of male and female rats received either glucose by stomach tube or subcutaneously as did the animals in Series I or II. However, they received no ethionine or saline until 3 hours after the initial dose of glucose when 3 animals were sacrificed and the remainder received either ethionine or saline in 2 divided doses as in Series I. These remaining rats were sacrificed 7 hours after the first dose of glucose. Three rats were used for each experimental and control group in Series I and II, and 9 animals were used in each experiment in Series III. The ethionine was dissolved in water to a concentration of 25 mg per ml. The glucose solution for subcutaneous injection contained 75 TR units of hyaluronidase per 100 ml to facilitate absorption. The animals were sacrificed by a blow on the head and the liver rapidly removed and weighed. Duplicate samples of the liver weighing approximately 1 g each were placed in tared 40 ml centrifuge tubes containing 3 ml of 30% KOH and the tubes were reweighed. Liver glycogen was isolated and hydrolyzed by the method of Good, Kramer, and Somogyi(8). Glucose was determined by Nelson's method(9). In some animals, residual glucose in the alimentary tract was determined at the time of sacrifice. The upper end of the esophagus and lower end of the rectum were tied with string and the alimentary tract between the ligatures was removed and placed in boiling distilled water. The tract was then opened and repeatedly washed with hot water. After cooling, the washings were diluted to an appropriate volume (500 or 1000 ml). To 10 ml of this suspension were added 5 ml of 0.3 N Ba(OH)₂, 5 ml of 5% ZnSO₄, dilute Na₂SO₄ solution to remove excess Ba⁺⁺ and water to 50 ml. After filtration, 1 ml aliquots were removed for glucose determination by Nelson's method(9).

Results. In Series I, female rats injected with ethionine had virtually no liver glycogen 6 hours after the administration of 2.5 g of glucose by stomach tube (Table I). Male rats showed more glycogen than females but still much less than the control animals. It

TABLE I. Influence of Ethionine upon Hepatic Glycogen and Residual Alimentary Glucose following Glucose by Stomach Tube (Series I).

Treatment	Sex	No. of animals	Liver glycogen		% diff. from control*	Glucose in alimentary tract		
			1 Per 100 g wet liver, g	2 Per liver/100 g body wt, mg		No. of animals	Total, mg	% unab-sorbed
S†	♀	8	2.74 ± .16†	82.6 ± 4.9†		5	108 ± 28†	4.3
E	♀	6	<.05	1.3 ± .1	-98	3	669 ± 64	26.8
M	♀	5	2.71 ± .25	81.1 ± 5.3		3	1082 ± 29	43
E-M	♀	9	1.90 ± .5	60.9 ± 6.8	-25	6	1163 ± 153	47
S	♂	8	1.84 ± .21	52.5 ± 5.1		6	54 ± 17	2.2
E	♂	9	.75 ± .17	20.8 ± 5.2	-60	6	358 ± 34	14.3
M	♂	6	2.67 ± .19	80.8 ± 6.0		3	475 ± 264	19
E-M	♂	6	1.80 ± .19	56.7 ± 11.5	-30	3	1475 ± 118	58

* Figured on column 2. Saline group is the control for the ethionine group and the methionine is control for the ethionine-methionine.

† S = Saline; E = Ethionine; M = Methionine.

‡ Stand. error of mean.

was noted that, at the time of sacrifice, the stomachs of the ethionine-treated females were filled with fluid while those of the controls were empty. The experimental males likewise showed fluid in their stomachs but less than the females. It was therefore thought possible that ethionine might be preventing glucose from reaching the liver by interfering with the passage or absorption of glucose in the alimentary tract. To test this, the glucose remaining in the alimentary tract at the end of the 6-hour experimental period was determined in many subsequent experiments. It was found that approximately 25% of the administered glucose remained unabsorbed in females given ethionine while only about 4% remained in the control animals (Table I). Male experimental rats showed about 14% unabsorbed glucose as compared to about 2% in the controls. The simultaneous occurrence

of inhibition of liver glycogen and decrease in the glucose absorption suggested that the interference with glucose absorption might be an important factor in the decreased hepatic glycogen formation in animals injected with ethionine.

The experiments in Series II were conducted to test this possibility. To overcome the possible effect of diminished glucose absorption on liver glycogen formation, the glucose was administered subcutaneously instead of by stomach tube. In these experiments, ethionine-treated females still showed much less liver glycogen than control females (Table II). The difference between the sexes was less than in the first series.

TABLE II. Influence of Ethionine upon Hepatic Glycogen following Subcutaneous Administration of Glucose (Series II).

Treatment	Sex	No. of animals	Liver glycogen		% diff.* from control
			1 Per 100 g wet liver, g	2 Per liver/100 g body wt, mg	
S†	♀	9	2.65 ± .35†	76.1 ± 10.4†	
E	♀	6	.74 ± .20	21.3 ± 6.2	-72
M	♀	6	1.81 ± .22	54.4 ± 5.5	
M-E	♀	3	1.34 ± .4	39.5 ± 5.1	-27
S	♂	5	2.34 ± .41	68.0 ± 15.3	
E	♂	6	1.03 ± .23	27.0 ± 5.8	-60

* See footnote (*), Table I.

† S = Saline; E = Ethionine; M = Methionine.

‡ Stand. error of mean.

These results demonstrated that ethionine has a more direct effect on liver glycogen content in addition to its effect on the alimentary tract. However, the nature of this effect on the liver was in doubt. Conceivably, ethionine could inhibit synthesis of glycogen or could initiate a breakdown of preformed glycogen. A third series of experiments was therefore performed in order to help decide between these possibilities. Animals were given glucose either by stomach tube or subcutaneously and were allowed to synthesize liver glycogen in the absence of ethionine or administered saline. At the end of a 3-hour period one group was sacrificed and the remaining animals received ethionine or saline. The animals receiving glucose by stomach tube had at 3 hours approximately one-half the glycogen content of the controls sacrificed at 7 hours

TABLE III. Effect of Ethionine on Preformed Hepatic Glycogen (Series III).

Treatment	Sex	Liver glycogen, mg/liver/100 g body wt	
		3 hr*	7 hr*
S—st†	♀	36.8 ± 2.9‡(5)§	72.4 ± 7.1‡(6)§
E—st	♀		31.4 ± 9.6 (6)
S—st	♂	37.3 ± 2.8 (3)	75.4 ± .17(3)
E—st	♂		71.7 ± 5.1 (3)
S—sc	♀	58.3 ± 4.7 (6)	59.9 ± 7.8 (6)
E—sc	♀		30.6 ± 8.1 (6)
S—sc	♂	51.9 ± 8.5 (3)	48.7 ± 11.2 (3)
E—sc	♂		38.2 ± 8.4 (3)

* Fasted animals given glucose as indicated and liver glycogen determined on representative animals at end of 3 hr. At this time the remaining animals were treated with saline or ethionine and sacrificed 4 hr later or 7 hr after glucose admin.

† S = Saline; E = Ethionine; st = glucose by stomach tube; sc = glucose subcutaneously.

‡ Stand. error of mean.

§ No. of animals.

(Table III). At 7 hours, ethionine-treated females showed no mean rise over the value at 3 hours. However, the liver glycogen values were extremely variable in the experimental rats, some animals showing virtually no glycogen while others had amounts equal to the control animals. The control animals, on the other hand, had values close to each other. This suggested a breakdown of preformed liver glycogen in some animals treated with ethionine. This was confirmed in other experiments in which the glucose was administered subcutaneously. In this group, the liver glycogen during the early period increased more rapidly than in those given glucose by stomach tube but the level at the end of 7 hours was lower (Table III). The ethionine-treated females had liver glycogen values much below the control females. The males showed almost no effect of ethionine on liver glycogen. When glucose was administered to males by stomach tube, ethionine had no detectable influence on liver glycogen levels. With subcutaneous glucose, the male rats demonstrated a questionable decrease in liver glycogen.

Since ethionine is believed to owe some of its biological effects to an interference with methionine metabolism, the ability of methionine to reverse the effects on liver glycogen was tested. Methionine partially prevented the deleterious influence of ethionine on liver

glycogen, both when glucose was administered by stomach tube (Table I) and subcutaneously (Table II). However, the reversal was never complete with the dosage of methionine used (equimolar). Like ethionine, methionine when administered alone increased significantly the per cent glucose unabsorbed from the alimentary tract. Yet, despite the interference with glucose absorption, the liver glycogen values were not consistently less than those of the controls. Again, methionine plus ethionine caused the greatest interference with glucose absorption (ca 50%) but the liver glycogen values were higher than found with ethionine alone.

Discussion. It is evident from this study that ethionine has effects both upon glucose absorption from the alimentary tract and upon the glycogen content of the liver.

The interference with the function of the gastrointestinal tract is apparently not a unique property of ethionine, since methionine has the same effect and since the greatest inhibition was found when both amino acids were administered together. It is not clear from this study whether the action is primarily on motility of the gastrointestinal tract or on absorption or whether this effect is characteristic of this type of S-containing amino acid or is shown by other amino acids as well.

Ethionine may inhibit synthesis of liver glycogen from glucose, accelerate its breakdown or have both effects. The results of experiments in Series III suggest an effect primarily upon breakdown, since some female animals showed virtually no liver glycogen. However, since the glycogen content at any time is the resultant of the relative rates of the 2 opposing reactions, synthesis and breakdown, any inhibition of synthesis coupled with a normal rate of breakdown might account for the observed decreases in hepatic glycogen.

It appears probable that the observed decrease in liver glycogen with ethionine is at least in part the result of interference with some metabolic function of methionine, since methionine administration gave partial protection against the ethionine effect on liver glycogen. There is, however, no known metabolic function of methionine which can explain this action of methionine.

The sex difference observed in this study is much less striking than that found previously in studies on fatty liver and interference with protein metabolism. It is most clearly apparent in the experiments in Series III. Since there also appears to be a sex difference in the inhibition of glucose absorption by methionine, the differences between the sexes with ethionine might be predominantly due to an interference with alimentary tract function rather than with liver function.

Previous studies have shown that the fatty liver induced by ethionine may be prevented or cured by the administration of glucose or sucrose by stomach tube(1,10). From this study, it is clear that ethionine-treated female rats given glucose by stomach tube do not store much glycogen in the liver. The protective effect of glucose therefore is probably not mediated through increased hepatic glycogen formation. Possibly the metabolism of the excess neutral fat(2) in the livers of rats treated with ethionine is facilitated by glucose oxidation.

Summary. The intraperitoneal injection of DL-ethionine has been found to have the following effects on carbohydrate metabolism: 1) decrease in liver glycogen in both male and female rats given glucose by stomach tube 2) decrease in liver glycogen in male and fe-

male rats given glucose subcutaneously 3) increase in glucose unabsorbed from the alimentary tract in male and female rats given glucose by stomach tube and 4) decrease in liver glycogen in female rats given glucose by stomach tube or subcutaneously 3 hours prior to the administration of ethionine. Female rats generally showed changes of greater magnitude than males under the same conditions. Methionine prevented in part some of the effects on liver glycogen but had an effect similar to ethionine on glucose absorption from the alimentary tract.

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Nitrogen Mustard as a Teratogenic Agent in the Mouse.* (21207)

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While studying certain effects of nitrogen mustard on mice it was found that this substance is a powerful teratogenic agent when administered during the second week of pregnancy. Many of the anomalies produced are clear-cut and, within limits, predictable. In certain respects some of them are similar to those observed by Ingalls *et al.*(1) in the progeny of mice subjected to severe hypoxia and

by Russell(2) in young following x-ray treatment. They also resemble anomalies in rat fetuses found by Gillman *et al.*(3) after treatment with trypan blue and by Wilson(4) after azo blue. In gross manifestations the present anomalies include a wide spectrum of forms ranging from squat, edematous, exophthalmic embryos with practically no legs or tails, to those with defects so minor as to be detected only on the dissection of half grown young. Localized manifestations appear as hydrocephalus, cerebral hernia, abdominal hernia,

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eye and ear defects, hare lip, deficient lower jaw, curly tails and abnormal feet with either an excess or deficiency in digits. Especially interesting are very small, translucent embryos with conspicuous vascular trees and strong heart action. It appears that the exact time of treatment is an important factor in determining the kind of anomaly produced, and it seems probable that an analysis of any of these aberrant responses would throw a good deal of light not only on teratogenesis as such, but on factors involved in normal morphogenesis as well. Only effects registered in the feet are reported at this time.

Procedure. Hydrochloride of the "HN₂" form of nitrogen mustard, methyl bis (β chloroethyl) amine hydrochloride, was used throughout. In ordinary dosages this substance is very toxic to pregnant mice, usually causing rather prompt abortion, and even in amounts too small to produce noticeable clinical effects on the mother it may do permanent damage to the young *in utero*. The dosage that we have found most effective in the production of fetal anomalies is from one to 2 μ g of nitrogen mustard to a gram of pregnant mouse. This is administered on the 10th to 12th day of gestation as a single intraperitoneal injection of about .05 cc of a freshly prepared solution made by dissolving 10 mg of the dry crystals in 20 cc of .75% saline. The fetuses are then examined on the 14th or some subsequent day, or are allowed to be born and observed during the postnatal period.

Results. Following this procedure, some or all members of most litters are found to show aberrant development. The potential number of anomalies, however, is probably less than recorded because many embryos die immediately after the injection, and defective fetuses which go to term are less likely to survive the hazards of birth and maternal cannibalism. Nevertheless, there are a few litters that show no indication of loss and no abnormal young. While such litters must be counted in the totals, it is probable that some of them represent instances in which the injection (.05 cc) was not successfully administered or fell below an effective threshold. Individual and racial differences in response to treatment have become evident as the work progressed. In all groups

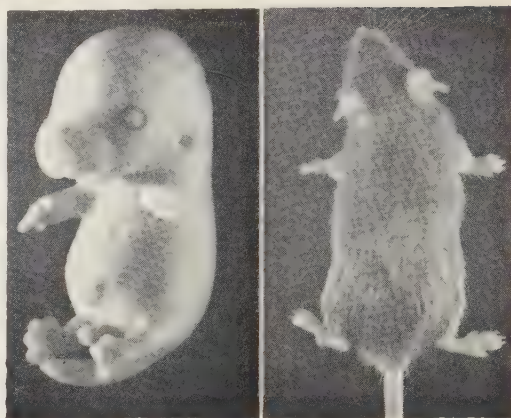


Fig. 1

Fig. 2

FIG. 1. A 14-day embryo obtained 3 days after treatment of the mother (an F₂ animal from a C3H \times J cross) with about 50 μ g of HN₂. Left front foot is a "peg" with no distinct toes; left hind foot has three toes (apparently II, III and IV); right hind foot, 4 toes, V being absent; right front foot has 5 toes, but I and II are somewhat fused with the former obscured in the photograph. Individuals in the litter from which the specimen came ranged from very defective to apparently normal.

FIG. 2. A somewhat less aberrant specimen at 9 weeks of age. Treatment was similar, except that only about 25 μ g of HN₂ was administered to the mother (S-br strain). In this case left front foot lacks digits I, IV and V, and left hind foot digit V. Both right feet are normal. The only litter mate recovered was a normal female.

the pattern of observed anomalies seems to indicate definite and consistent responses to an altered humoral environment rather than the haphazard effects of localized injuries.

Of 475 available young from 78 treated females, there were 178 abnormal specimens of which 150 (about 31% of the total) showed deficiencies in the feet. It appears that when reduction occurs, it usually involves one or more fifth digit, which may be slightly reduced, greatly reduced, or completely suppressed (Fig. 1). Next most susceptible to reduction or disappearance is digit I, and so on down to a single third digit or partially fused aggregate which give the end of the monodactyl limb a peg-like appearance. Deficiency in the digits is reflected in the morphology of the more proximal skeletal elements.

Of the 150 animals with deficiency in foot development the defects were limited to, or greater on, the left side in 64 cases and on the

right side in 2 cases, indicating that where there is a difference in the response of the 2 sides it is greater on the left perhaps 30 times as often as on the right. Fig. 1 and 2 show typical responses of this sort. A related point of interest is found in the evidence for a certain degree of autonomy for each of the limbs individually. In extreme cases all 4 limbs may be reduced to mere pegs, but if only one foot is reduced it is almost invariably the left front one. Next most often affected is the left hind foot, followed by the right hind foot, with the right front foot being lowest in both the grade and frequency of anomalies.

Discussion. Whether the nitrogen mustard can traverse the placenta and act directly on a growing embryo at a critical moment in its development, or can produce its effect only through some indirect chain of reactions, is not clear. In light of other recent findings, the latter possibility might seem the more probable, for it has become apparent that somewhat comparable results can be produced in a variety of ways. Hogan(5,6), Evans(7), and their coworkers have found that the expression of hydrocephaly and other anomalies in the rat can be regulated in some measure by diet and by an anti-folic acid agent; Gillman *et al.*(3), and Wilson(4) have produced fetal anomalies in rats by use of 2 out of a considerable number of tested dyes which are taken up by the reticuloendothelial system; Russell(2) produced them by x-ray and Ingalls *et al.*(1) by hypoxia. In their structural formulas the 2 effective dyes have points in common, but the similarities among agents thus far found to be effective seems to end at this point unless, as seems not unlikely, they all produce some degree of toxicity or anoxia which, catch-

ing the embryo at a critical moment, can permanently alter its course of development. Of special interest is the fact that an amount of nitrogen mustard too small to produce detectable results in the mother can profoundly influence the young and that, if needed for morphological studies, stages in the development of certain specific anomalies could be produced almost at will.

Summary. A single intra-abdominal injection of nitrogen mustard in an amount equivalent to about 1 μ g per g of body weight and administered on the 10th to 12th day of gestation has no apparent effect on a pregnant mouse, but may have a pronounced effect on the young *in utero*. Some of the latter are killed outright and others show a wide range of resultant anomalies. Only anomalies involving the feet are reported here. The grades and distribution of deficiencies in the feet reveal a significant difference in responses on the 2 sides of the body and a considerable developmental autonomy on the part of each individual foot.

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Production and Counteraction of a Fatty Acid Deficiency in the Guinea Pig. (21208)

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Most mammals are known to require a dietary source of unsaturated essential fatty acids. With fat, as such, also supplied, growth in some animals may be somewhat accelerated. In the guinea pig, however, neither a requirement for unsaturated fatty acids nor for fat has been previously demonstrated. The present investigation is concerned with a study of these problems.

Methods. Three experiments have been conducted, each with 16 animals of the Hartley strain, 2 to 4 days of age, with an average weight per group of 101 or 102 g and with an equal number of males and females. Half of the animals were placed on the basal semi-synthetic diet previously described(1) containing 30% vitamin-free casein, 4 types of carbohydrates, 6% salts, 7.3% fat (corn oil), and liberal amounts of the known vitamins. The other half received the diet with fat omitted and cerelose substituted. The fat-soluble vitamins were added in alcohol solutions.

Results. After subsisting on the diet 25 to 30 days, the guinea pigs on the fat-free diet began to show a retardation in growth, with the differences in weight as compared to the control animals becoming greater with increasing length of time on the experiment. Table I shows the results of the 3 experiments.

After a period of 6 to 8 weeks (room atmosphere had low average humidity) on the fat-free diet many of the guinea pigs developed dry scaly dermatitis on the ears and feet

which increased in severity with time. All showed severe drying of the inside of the ears. Marked scaliness was later observed over the entire body and superficial ulcers developed on some of the animals, apparently as a result of scratching. Sensitivity to touch and restlessness were notable. Some of the animals showed loss of hair from the abdomen and inner surface of the legs. The feet of some of the animals appeared to be swollen and bluish, and in some cases a cyanotic condition developed. Marked daily fluctuations in weight of the deficient animals were frequently observed. It seems unlikely that pyridoxine deficiency was involved in the production of the syndrome since the diet contained 16 mg pyridoxine per kg. The control animals showed none of the above symptoms.

Therapeutic trials were made with one series of animals. One of the guinea pigs, in a critical state as a result of the deficiency, was placed on the complete diet containing corn oil. Within 2 days improvement was observed and eventually there was full recovery. Three of the other severely deficient animals were treated for a period of one week with daily oral doses of linoleic acid (10, 30, and 60 mg doses, respectively). The feet of the animals at the 2 higher levels of dosage lost the bluish tinge and acquired a pink color, the dermatitis disappeared, and growth was somewhat accelerated. Toward the end of the one-week period of dosage even the animals on the 10-mg level showed improvement, particularly with respect to the dermatitis. Table II summarizes the changes in weight during a 4-week period following the treatment with linoleic acid.

At the start of the treatment the deficiency of the guinea pig receiving the 30 mg supplement was somewhat more advanced than that in the animals receiving the 10 and 60 mg doses, respectively. There was no doubt, however, about the beneficial effect of linoleic

TABLE I.
Effect of Dietary Fat (Corn Oil) on Growth.

Exp.	Time on diet (days)	% fat in diet	Wt gain (g)	Survivors*
I	72	0	277 ± 25	7
		7.3	405 ± 26	8
II	125	0	377 ± 45	6
		7.3	573 ± 43	8
III	161	0	427 ± 39	6
		7.3	690 ± 42	6

* 8 animals started per group.

TABLE II. Effect of Linoleic Acid on Growth of Fat-Deficient Guinea Pigs.

Supplement (mg daily for 1 wk)	Wt (g)		Total gain (g)			
	1 week before treatment	Time of starting treatment	Wks after start- ing treatment			
			1	2	3	4
10	462	477	3	23	31	30
30	538	534	12	18	25	40
60	486	486	14	51	69	80
0	624	655	0	0	-12	4
0	576	587	8	12	15	6

acid in all 3 of the animals. Improvement in the animal which received the 10 mg dosage appeared to have subsided by the end of the third week. Eventually deficiency symptoms reappeared in all of the supplemented animals but latest in the one treated with the highest dosage of linoleic acid.

Summary. These experiments have shown

that omission of fat from the diet of the guinea pig results in a syndrome characterized by: retardation of growth, dermatitis, marked drying of the inside of the ears and, in some animals, ulcers, loss of fur, and a tendency to a swollen, somewhat cyanotic condition of the feet. All of these symptoms were corrected by the oral administration of linoleic acid. These results afford the first demonstration of the necessity for the guinea pig of a dietary source of linoleic acid or, possibly, of some other fatty acid present in corn oil.

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Lipemia Clearing by Hyaluronidase, Hyaluronate, and Desoxycorticosterone, and its Inhibition by Cortisone, Stress, and Nephrosis. (21209)

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The lipemia clearing action of heparin(1) has been amply confirmed. A similar action has been reported for other compounds(2,3) but has been established with certainty only for sulfated polysaccharide anticoagulants (2,4,5). The apparent specificity of the type of compound that caused lipemia clearing and the anticlearing action of protamine and anti-heparin dyes have suggested to some observers that heparin has a physiologic role in the transport and metabolism of lipids(5-9).

We have reported that hyaluronidase (H) has antihypercholesterolemic action(10). It was of interest therefore to determine whether it would also clear lipemia. Since in our studies of the ground substance(11-13) partially depolymerized hyaluronic acid (PDHA) and desoxycorticosterone (DCA) had effects resembling those of H while cortisone and stress were opposite in effect, it was of further interest to ascertain whether the same relationship prevailed with respect to lipemia clearing.

*Materials and methods.** Purified hyaluronic acid (HA) and PDHA were the same as that used in our previous studies(14). The HA had 3.4% N, less than 0.01% carbohydrate by the anthrone method(15,16) and no P, S, or halogen. The bovine testicular H (6000 TRU/mg N) used in all tests had 1.9 U proteolytic activity/mg by the method of Hadidian(17). Higher potency H (28000 TRU/mg N) was used to inactivate the lipemia clearing factor (CF) *in vitro* in order to eliminate significant proteolytic activity (less than 0.005 U/test). Sodium alginate was prepared by Dr. Harvey E. Alburn and was fractionated to yield a product with a molecular weight of 13,000-20,000 to approximate that of heparin. Plasma was decanted from citrated blood (0.1 ml M/10 Na citrate/ml) centrifuged at 2000 RPM until separation oc-

* All compounds were dissolved in 0.9% NaCl except DCA which was in oil (Cortate, Schering) and cortisone which was a 0.5% suspension of crystalline acetate in 0.5% acacia.

TABLE I.
Effect of Plasma from Treated Rats on Light Transmission of Lipemic Dog Plasma (LP).

Rat treatment	Route	mg/kg	LP	Avg % light transmission (LP + rat plasma min. after inj.)				
				60	120	180	240	300
Heparin	IV	4	24	86	84	85	45	43
"	PO	50	26	32	34	25	30	28
H	SC	150 TRU	27	69 ⁴	85 ⁴	86	97 ⁵	86 ⁴
PDHA	IV	10	29	89	86	88	86	84
"	SC	50	29	77 ⁵	98	79	54	29
"	PO	50	18	78	93	70	54	25
HA	IV	25	16	72 ⁵	54	46	30	20
DCA	IM	5	30	41	28 ⁵	64	91	77
Cortisone	IM	5	10	9 ⁵	10 ⁴	10 ⁵	10 ⁵	10 ⁵

Transmission values are averages of 6 rats except where superscripts indicate smaller groups.

curred, immediately stored at 5°C, and used only on the day of collection. Hemolyzed plasma was discarded. Rat blood was collected from the heart exposed under light ether anesthesia. Lipemic plasma (LP) was from dogs receiving 20-25 ml cottonseed oil (CSO)/kg intragastrically and was considered satisfactory if the light transmission did not exceed 30%. Light transmission was read at 650 μ in a Beckman quartz spectrophotometer using silica microcuvettes. Standardization was against distilled water. All readings were made at 22°C. In most instances the effect of a compound on lipemia was determined in a 2-stage test(18,19) by incubating 1.0 ml LP with 0.1 ml rat plasma for 5 minutes at 22°C and recording the change in light transmission. Each of the following compounds was administered to 15 male and 15 female albino rats weighing approximately 130 g: heparin intravenously and orally, 4 and 50 mg/kg, respectively; H subcutaneously, 150 TRU; HA IV, 25 mg/kg; PDHA IV, 1, 5, 10, 25, and 50 mg/kg; PDHA SC and PO, 25 and 50 mg/kg, respectively; DCA intramuscularly, 5 mg/kg; and cortisone acetate IM, 5 mg/kg. All oral administrations were in fasted rats. Groups of 3 male and 3 female rats were killed at hourly intervals thereafter for determination of plasma clearing activity. The effect of PDHA (10 and 20 mg/kg IV, and 50 mg/kg PO), HA (25 mg/kg IV) and alginate were also tested in dogs. The course of lipemia induced in fasted dogs was established for 6 hours in 3 males and 3 females. Lipemia was repeatedly induced at 3-4 day intervals for the purpose of testing the effect

of a compound or for restandardization. When PDHA was tested for efficacy by the oral route the dogs did not receive CSO; instead the plasma taken at hourly intervals was added to lipemic plasma *in vitro* in the ratio of 0.1 ml:0.5 ml. Plasma from the following groups of rats was tested for ability to inhibit (CFI) the clearing action of plasma (CF) from rats receiving 4 mg heparin/kg IV: one hour after cortisone (5 mg/kg IM); 24 hours after exposure to 2°C; 14 days after nephrotic syndrome was induced by administration of anti-kidney serum (AKS). CFI from cortisone was also tested against CF 3 hours after DCA (5 mg/kg IM) and one hour after PDHA (10 mg/kg IV). LP (2.0 ml) was incubated 5 minutes at 22°C with 0.1 ml CF + 0.1 ml CFI. Heat stability of CFI was determined by heating on a water bath for one hour at 60°C and adding to LP + CF. Susceptibility of CF to inactivation by H was determined by incubating plasma obtained one hour after 4 mg heparin/kg IV with 10 TRU H/ml at 36°C for one hour. Aliquots were withdrawn every 5 minutes and tested against LP. Average potency H was used in one test and high potency H in another.

Results. Table I lists the various compounds that were administered to rats for determining the effect on LP. The degree of lipemia of the control plasma used for testing the particular compound is also listed. The CF activity of plasma samples taken from rats sacrificed at the times indicated after injection of the compound is indicated as per cent transmission. Since normal plasma gave transmission readings of 85% or better, LP (30% or

TABLE II. Effect of Hyaluronate and Alginate on Lipemia in Dogs.

	—% transmission (avg 6 dogs) min. after CSO orally—									
	Pretreat- ment	60	90	120	150	180	210	240	300	360
Lipemia control (standardization)	93	36		21		29		35	25	46
PDHA IV (20)†	70	55*	77	85		88		59	46	37
PDHA IV (10)	94	55*	92	92	84	64		52	41	8
Restandardization	89	38		32		28		40	44	48
Alginate IV (50)	92	48*	16	17		18†	42	56	61	
Restandardization	80	33		28		18		30	32	36
PDHA PO (50)	86	66		87		95		64	46	
LP	87	43		14		17		36	37	

* Compound administered.

† HA administered.

‡ () mg/kg.

less) was considered to be cleared when light transmission approximated these. Each clearing value listed in Table I is the average of 6 rats except where smaller groups are indicated by superscripts. When pronounced lipemia clearing was present, transmission above 60% had a S.D. of $\pm 4-6\%$ and below 40% the S.D. was $\pm 10-20\%$; the increase in the S.D. can be attributed, in part, to the shorter sojourn of the CF in some animals than in others. When no lipemia clearing occurred the S.D. was $\pm 4-6\%$. In addition to confirming the effectiveness of intravenous heparin the data show that H, PDHA, and DCA released CF into the plasma. HA appeared also to have this property but to a much lesser degree. Cortisone did not release CF. Release of CF following oral administration of PDHA is in contrast to the ineffectiveness of orally administered heparin.

The values in Table II are averages obtained on 6 dogs and show 1) the lipemia clearing action of parenteral PDHA and HA in dogs receiving large doses of CSO by stomach tube, 2) the failure of alginic acid, a plant polyuronic acid, to exert such action, and 3) the effectiveness of PDHA when administered orally.

Incubation of CF with medium and high potency H preparations (10 TRU/ml) at 36°C resulted in progressive loss of clearing activity. With both preparations the decrease was 20% in 15 minutes of incubation, 50% in 25 to 30, and 100% in 45 minutes. CF maintained at this temperature for 60 minutes had no loss of activity. Table III lists data showing that cortisone treatment, stress, and the nephrotic syndrome inhibited the lipemia clearing action of CF plasma. Dilution of

CFI with physiological salt solution decreased the inhibitory action. The inhibitor was stable when heated at 60°C for one hour.

Discussion. Our chief interest was in establishing the effect of H, HA, PDHA, DCA, cortisone, stress, and nephrosis on lipemia clearing. The method employed is quantitative for establishing the potency of a compound with respect to total clearing and for duration of action. These studies reinforce decisively the current concept that lipemia clearing is unrelated to anticoagulant action because of all the compounds used only heparin prevents clotting. The data establish that CF activity may be completely abolished by CFI. They are not consistent with the growing concept that heparin is the physiologic agent responsible for lipemia clearing. It appears that mucopolysaccharides of the ground substance or agents that activate them may release CF; conversely, those agents that depress their activity may release CFI. Thus H and DCA which increase permeability of the ground substance by depolymerizing HA also released CF; cortisone and stress, which have opposite effects on the ground substance released CFI. Alginate of the same molecular weight as heparin did not release CF, although in some respects it resembles mucopolysaccharides, the chief difference being that it does not contain amino sugar residues.

The fact that subcutaneous injection of H released CF *in vivo* is not inconsistent with the finding that H inactivated CF *in vitro*. The amount of H injected subcutaneously would not achieve an adequate concentration in the blood to duplicate the *in vitro* results. It is more likely that injection resulted in the formation of PDHA which released CF. It

TABLE III. Effect of CF and CF + CFI Rat Plasma on Lipemic Dog Plasma (LP).

No. rats CF	Source of CF	No. rats CFI	Source of CFI	—Avg % light transmission—		
				LP	LP + CF	LP + CF + CFI
6	Heparin	15	Cortisone	9	96	10
6	PDHA	6	"	22	86	22
6	DCA	6	"	20	97	21
6	Heparin	6	Stress	20	96	21
6	"	4	.1 AKS*	18	86	41
6	"	3	.2 "	23	86	27
6	"	5	.4-.8 "	21	86	23

* ml anti-kidney serum/100 g of rat.

CF = Lipemia clearing factor.

CFI = " " " inhibitor.

has been established that CF is readily destroyed by heat, high concentrations of salt, and chymotrypsin digestion. It has also been established that CF contains a lipoprotein which requires a non-lipoprotein co-protein in order to produce clearing(8). H is not known to act on lipoproteins and it may be assumed that it may have hydrolyzed a mucopolysaccharide moiety of the co-protein. It is thus indicated that it is not likely that heparin is the mucopolysaccharide attached to CF. It has already been pointed out that the proteolytic activity of the H used can be considered as negligible.

The finding that an inhibitor of lipemia clearing was released by cortisone, stress, and renal disease is of considerable importance. The effect of cortisone and stress indicates that release of CFI is another manifestation of the general adaptation syndrome. CFI was present in all rats manifesting the nephrotic syndrome but only those in the more severe phases had gross lipemia in addition. The question is raised whether development of pathological lipemia is preceded by increase of inhibitor to a critical level necessary to completely suppress clearing. CFI effectively neutralized considerable amounts of CF and it therefore remains to be established whether administration of heparin in non-anticoagulant doses and of other CF releasing agents would be of benefit in the treatment of pathological lipemia.

The mechanism by which CFI suppresses CF is not known. The heat stability suggests that CFI is not a complex protein.

Summary. 1. Partially depolymerized hyaluronic acid released a lipemia clearing fac-

tor following intravenous, subcutaneous and oral administration to rats. Hyaluronidase and DCA which were administered only to rats hypodermically were also effective. 2. Plasma from rats treated with cortisone prevented the lipemia clearing by heparin, DCA and partially depolymerized hyaluronic acid. The effect of exposure to cold was tested only against heparin lipemia clearing and was also found to inhibit it. 3. Release of a lipemia clearing inhibitor appears to be another manifestation of the general adaptation syndrome. 4. Plasma from rats made nephrotic by administration of low doses of anti-kidney serum also had an inhibitor of the clearing factor, and those given larger doses of anti-kidney serum had in addition gross lipemia suggesting that pathological lipemia may be preceded by the appearance of the inhibitor. 5. The lipemia clearing factor was inactivated by incubation with hyaluronidase *in vitro* suggesting that this factor contains a mucopolysaccharide moiety which is not heparin. 6. The activity of the inhibitor of the lipemia clearing factor was not affected by heating at 60°C for one hour.

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Failure of Newborn Rat to Respond to Hypoxia with Increased Erythropoiesis.* (21210)

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It is known that the newborn young of many mammals have a normal or higher than normal erythrocyte count and hemoglobin(1) and that these values decrease immediately after birth, reaching minimal and subnormal levels within a matter of days or weeks depending on the species. One of the proposed explanations of this neonatal anemia is that in intra-uterine life the fetus exists under reduced oxygen tension, to which it responds by active erythropoiesis(2). After birth the newborn, having adequate oxygen, no longer requires the excess of red cells formed during intra-uterine life. Red cell production decreases somewhat, the physiological anemia of the newborn supervenes, and the count only gradually returns to a stable, normal level.

If the hypothesis were sound that hypoxia is the direct determining factor for the high count at birth and, therefore, indirectly responsible for the ensuing anemia, then continued hypoxia after birth should prevent the neonatal anemia or perhaps even result in polycythemia. It is the object of this paper

to report the hematological changes found in the newborn rat when exposed to low oxygen tension, as compared with the well-known erythropoietic response of older rats to hypoxia.

Materials and methods. Rats of the Long-Evans strain were used throughout these experiments. Two litters of 6 male rats each were divided into 2 groups; 3 were chosen from each litter. One group, 6 rats, was exposed in a large decompression chamber to a simulated altitude of 15,000 feet for 6 hours a day from the 4th to the 18th days of age. At the end of each exposure, the young were returned to their own mothers. The remaining 6 rats, which served as controls, were also removed from their mothers over the same period daily for 6 hours. The temperature of the chamber was maintained at $25^{\circ} \pm 2^{\circ}\text{C}$. The relative humidity was in excess of 95% throughout the experiment. To further protect the young from chill and desiccation, they were placed in a small feeding can in a deep well of shavings, which simulated a nest and prevented their scattering. The controls, during the period when they were separated from their mothers, were similarly protected. The nursing mothers were maintained on a

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TABLE I. Comparison of the Erythropoietic Response of Newborn Male Rats Exposed 6 Hours Daily for 14 Days at 15000 Feet with that of Adult Male Rats Similarly Treated.

Age group, days	Altitude, ft ($\times 1000$)	No. of rats	Body wt, g	Hemoglobin, g/100 ml	Hemoglobin/100 g body wt, g	Hematocrit, %	Red blood cell vol/100 g body wt, ml
4-18	S*	6	42 \pm .7	6.7 \pm .2†	.47 \pm .01	24.1 \pm .6	1.61 \pm .06
4-18	15	6	39.7 \pm .7	7.3 \pm .1	.51 \pm .03	24.2 \pm .4	1.67 \pm .04
90-104	S	6	304 \pm 10.0	13.5 \pm .3	.74 \pm .04	44.4 \pm .8	2.39 \pm .10
90-104	15	5	301 \pm 7.0	14.4 \pm .2	.90 \pm .03	47.7 \pm .7	2.96 \pm .05

* S = Sea level.

$$\dagger \text{Stand. error} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

stock diet known to be adequate for lactation.† The particular mothers chosen for the experiment had an excellent record for milk production. The newborn young, while still hairless, were checked daily, by direct inspection, for the presence of milk distended stomachs. Milk could still be seen in the stomach at the end of the 6-hour period in the chamber. Three unsuccessful attempts to perform this experiment were conducted before these necessary precautions were established. Records of body weight were kept for all the newborns and no significant changes in body weights were found between the animals exposed to the simulated high altitude and their respective controls (Table I, II). At the end of the experiment, determinations of red cell volume were made using the Fe⁵⁹ tagged cell dilution method(3). Hemoglobin determinations were done using Turner's method(4). Parallel determinations of these values were made in adult (90-day) rats for comparison of the response to hypoxia. Other groups of newborn rats were exposed daily for 6 hours to a simulated altitude of 22000 feet from the 4th to the 16th day of life. This type of intermittent stimulation to hypoxia (corresponding to that at 15000 or 22000 feet altitude) was known to cause an erythropoietic response in normal rats similar to, or even higher than that resulting from continuous exposure to hypoxia(5).

Results. It can be seen from Table I that exposure of the suckling rat to hypoxia from

the 4th to the 18th day of life, at 15000 feet, did not result in stimulation of erythropoiesis as measured by an increase in the hemoglobin, hematocrit or red cell volume. The adequacy of this stimulus to erythropoiesis in older animals is also shown in Table I. Similarly, exposure to a simulated altitude of 22000 feet from day 4 to day 16 did not stimulate erythropoiesis (Table II). It should be noted that these degrees of hypoxia were not even sufficient to prevent the development of neonatal anemia. At the 16th day of life (or the 18th, when the neonatal anemia is most severe) the decrease in the hematological values of hypoxic rats was the same as those of their normal age controls.

When it was found that rats did not respond to hypoxia in this neonatal period, other groups of young, growing rats were exposed to hypoxia for similar periods. Table II shows the hematological data from rats in which hypoxia was instituted at progressively older ages, from the 18th day to the 68th day of life. Rats in this age range all responded with significant increase in hematocrit, hemoglobin and red cell volume (Table II). In fact, the increase when hypoxia was instituted at 18 days of age was as great as in adults under the same stimulus and resulted in polycythemia.

Discussion. The failure of the rat, in the earlier neonatal period, to respond to hypoxia with an increased erythropoiesis makes it improbable that hypoxia is the direct stimulus to red cell production which leads to the high values found at birth. It is evident that termination of the hypoxia at birth could not, therefore, explain the post-natal anemia or the subsequent gradual recovery from this anemia.

† Diet I consists of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.0% fish oil, 0.75% NaCl, 1.5% CaCO₃, KI added (analysis 1 μ g iodine per g diet).

TABLE II. Erythropoietic Response of Male Rats of Different Ages Exposed Daily to an Altitude of 22000 Feet for 6 Hours.

Age group, days	Altitude, ft ($\times 1000$)	No. of rats	Body wt, g	Hemoglobin, g/100 ml	Hemoglobin/100 g body wt, g	Hematocrit, %	Red blood cell vol/100 g body wt, ml
4-16	S*	5	31 \pm 1.0	8.8 \pm .1†	.53 \pm .02	29.0 \pm 1.4	1.67 \pm .04
	22	6	33 \pm 0.9	8.3 \pm .4	.52 \pm .01	29.4 \pm .6	1.75 \pm .04
18-30	S	4	84 \pm 2.0	12.2 \pm .3	.81 \pm .01	39.3 \pm 1.3	2.59 \pm .10
	22	6	72 \pm 2.0	14.1 \pm .7	1.07 \pm .01	46.9 \pm 1.0	3.38 \pm .10
24-36	S	5	95 \pm 2.0	11.6 \pm .4	.74 \pm .01	39.1 \pm 1.2	2.41 \pm .05
	22	5	91 \pm 3.0	15.8 \pm .3	1.10 \pm .03	44.8 \pm 1.4	3.10 \pm .20
40-52	S	5	144 \pm 3.0	14.8 \pm .1	.78 \pm .01	46.9 \pm .5	2.44 \pm .08
	22	5	145 \pm 5.0	15.4 \pm .1	1.08 \pm .03	53.0 \pm 1.5	3.28 \pm .20
68-80	S	4	249 \pm 3.0	13.6 \pm .2	.66 \pm .02	44.1 \pm 1.5	2.25 \pm .10
	22	5	275 \pm 2.0	15.7 \pm .3	.95 \pm .03	49.9 \pm 1.6	3.02 \pm .10

* S = Sea level.

$$\dagger \text{Stand. error} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

Hypoxia before birth might be a contributing factor, but additional assumptions would still be necessary, such as: a substance from the mother passes the placenta and stimulates the fetal marrow; further, that this substance is not produced by the fetus itself immediately after birth in adequate amounts to maintain erythropoiesis at the birth level.

A pituitary fraction has recently been described which stimulated erythropoiesis in the hypophysectomized, adrenalectomized and normal rat(6-10) which is also able to prevent the development of neonatal anemia when injected into the newborn rat(11,12). Since it is known that the pituitary does not produce or release some of the known trophic hormones in the early period of life, it is not impossible that lack of production or release of the erythropoietic factor, during the neonatal period, results in the development of neonatal anemia and in the failure of the newborn rat to respond to hypoxia. The implication that the pituitary is at least in part responsible for the erythropoietic response to hypoxia is supported by the fact that hypophysectomized rats give an impaired response to hypoxia as compared with normal animals(5).

Summary. Suckling rats, between the age of 4 to 16 days, do not respond to hypoxia with an increased erythropoiesis as judged by increased hematocrit, hemoglobin and red cell volume.

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Effects of Cortisone and Antibiotics on Lethal Action of Endotoxins in Mice.* (21211)

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The administration of ACTH or cortisone to humans and animals enhances the severity of many natural or experimental infections, presumably by depressing the antimicrobial defenses of the host(1-3). On the other hand, it has also been shown that ACTH or cortisone may exert an "antitoxic" action *in vivo* against the lethal effects of endotoxins derived from a wide variety of gram negative bacteria(4,5). In a few natural infections, notably typhoid fever and acute brucellosis, where the endotoxins of the infecting microorganisms are thought to play an important role in the pathogenesis of the disease, the judicious use of cortisone or ACTH in the therapeutic regimen results in a rapid suppression of "toxicity" and an improvement in the sense of well-being of the patient(6-8). However, Spink and Hall(8) have strongly cautioned against the indiscriminate use of these hormones in human brucellosis, by pointing out the undesirable side effects which may occur and also because antibiotics alone provide effective treatment in the majority of these cases. This paper summarizes experiments on the protective effects of cortisone and the combined effects of cortisone and antibiotics in animals injected with lethal amounts of endotoxins derived from a coliform bacillus.

Methods and materials. The crude endotoxin used in this study was prepared from the cells of *Escherichia intermedium* as described elsewhere(9). A single batch of endotoxin was used in all experiments. It was stored in a dry state in a desiccating jar and made up to the desired concentration with sterile water on the day used. Standardization of the endotoxin dosage on an LD₅₀ basis proved to be impractical. Instead, sufficient endotoxin was injected into mice intraperitoneally in a vol-

ume of 0.5 ml to result in death of 80 to 100% of the animals within 48 hours. White Swiss mice weighing approximately 20 g and of heterogenous stock were employed. All animals were carefully observed for 48 hours after the injection of test materials and only deaths occurring within this period were recorded. Suspensions of cortisone acetate were supplied by Dr. E. Alpert of Merck and Co. Dilutions of the drug to the desired concentrations were made in sterile saline immediately before use. The cortisone was usually injected subcutaneously in a volume of 0.2 ml. The antibiotics used in this study were streptomycin calcium chloride complex, Merck, lot 2247; chloramphenicol, synthetic, Parke Davis, lot 151773, chlortetracycline, Lederle, lot 7-8557, and penicillin G, Bristol, lot G0426. The chloramphenicol was supplied by Dr. G. Rieveschel and the other antibiotics were obtained commercially. All solutions were made up fresh with sterile saline before each experiment and stored in the refrigerator until used. All antibiotics were injected subcutaneously in a volume of 0.2 ml, with the exception of chloramphenicol, which was injected in a volume of 0.5 ml.

Results. Protection by cortisone against lethal effects of endotoxin. A single 5 mg subcutaneous dose of cortisone injected one hour prior to or simultaneously with an intraperitoneal lethal dose of coliform endotoxin consistently protected from 60 to 90% of the animals from death. There was no protection, however, when the cortisone was injected one hour after the endotoxin, as shown in Table I. The route of injection of either the cortisone or the endotoxin could be varied without appreciably changing the results as long as the cortisone was given first. Thus, equally good protection was obtained when the cortisone was given subcutaneously and the endotoxin intravenously one hour later. There were no protective effects from the fluid in which the cortisone was suspended.

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TABLE I. Time Relationships of Cortisone Administration on Lethal Action of Coliform Endotoxins in Mice.

Materials injected*			Deaths		P values
1 hr before endotoxin	0 hr	1 hr after endotoxin	D/T†	%	
C	E	Sa	3/13	23	>.01
Sa	E + C	Sa	2/13	15	
Sa	E	C	10/12	83	
Sa	E	Sa	10/12	83	
C diluent only	E	Sa	5/5	100	

* C = Cortisone—5 mg inj. subcut. in a vol of .2 ml. E = Endotoxin—5 mg inj. intraper. in a vol of .5 ml. Sa = Saline, cortisone diluent—.2 ml inj. subcut.

† No. of animals dead/total No. of animals inj.

Effects of antibiotics with and without cortisone. Levaditi, Vaisman, and Reine(10) reported that in addition to its antimicrobial activity, certain sulfonamides were also able to exert an "antitoxic" action *in vivo* by protecting animals from a lethal injection of meningococcal endotoxin. These findings were confirmed by Carpenter *et al.*(11), and subsequently extended to show that penicillin (12,13), chlortetracycline, oxytetracycline, and chloramphenicol(14) also may be protective under certain conditions. A possible clue to the mechanism of this protection was noted in the bacteremia† which occurred in some animals a few hours after the injection of the endotoxin. It was postulated that the bacteremia might be due to an alteration of the intestinal mucosa by the endotoxin, and that the systemic invasion of the animal by a variety of organisms from the intestinal tract might contribute to the pathogenesis of the reaction. Experiments were therefore carried out to determine whether antibiotic therapy, by suppressing the bacteremia, would protect mice from an otherwise lethal dose of endotoxin. Of the antibiotics tested, which in-

† The bacteremia produced in animals following injection of endotoxin was first called to our attention by Dr. A. I. Braude(15), who noted such occurrences in mice injected with coli and aerogenes endotoxins. Bacteremia occurs sporadically and varies from 2 or 3 up to 2000 bacteria/ml of blood. On the basis of preliminary work, it does not appear likely that the bacteremia plays an important role in causing death, but studies on this point are continuing.

cluded chlortetracycline, penicillin, chloramphenicol, and streptomycin in a wide range of doses, only streptomycin in doses of 2 mg per mouse occasionally gave some protection, but the results were not reproducible. The possibility was considered that although streptomycin alone was not able to protect the animals consistently from the endotoxin, it might "reenforce" the protection given by cortisone and thereby increase the survival rate over that obtained with cortisone alone. However, just the contrary effects were produced. When streptomycin was injected one hour after the endotoxin and 2 hours after the cortisone, significantly higher mortality rates were obtained than with cortisone alone. The combined results from 3 experiments in which cortisone was administered in doses ranging from 1.2 to 5 mg and streptomycin in doses of 0.5 to 2 mg per mouse are shown in Table II. The results in each of the experiments were approximately of the same order, irrespective of the dosage of either agent employed. Thus, the average mortality rate in the group treated with cortisone was 11% as compared to 84% in the untreated controls. Streptomycin alone had little or no influence on the mortality rate but when it was injected into groups previously treated with cortisone, the mortality rate rose from 11% to an average of 45%. This "interference" by streptomycin with cortisone protection was demonstrable only when the antibiotic was injected after the cortisone; the simultaneous injection of both drugs one hour before the endotoxin gave results which

TABLE II. Effects of Streptomycin on Cortisone Protection against Coliform Endotoxin in Mice.

Materials injected*			Deaths		P values
1 hr before endotoxin	0 hr	1 hr after endotoxin	D/T†	%	
Sa	E	Sa	42/50	84	>.001
C	E	Sa	12/105	11	
C	E	S	54/119	45	
Sa	E	S	43/56	77	
C	Sa	Sa	0/15	0	
C	Sa	S	0/15	0	
Sa	Sa	S	0/15	0	

* E = Endotoxin—5 mg inj. intraper. in a vol of .5 ml. C = Cortisone—1.2 to 5 mg inj. subcut. in a vol of .2 ml. S = Streptomycin—.5 to 2 mg inj. subcut. in a vol of .2 ml. Sa = Saline—.2 ml inj. subcut.

† No. of animals dead/total No. of animals inj.

TABLE III. Effects of Penicillin on Cortisone Protection against Coliform Endotoxins in Mice.

Materials injected*			Deaths		P values
1 hr before endotoxin	0 hr	1 hr after endotoxin	D/T†	%	
Sa	E	Sa	33/34	97	>.001
C	E	Sa	12/35	34	>.01
C	E	P	34/50	68	>.001
C + P	E	Sa	9/35	26	
Sa	E	P	19/19	100	
Sa	Sa	P	0/10	0	
C	Sa	P	0/10	0	

* E = Endotoxin—5 mg inj. intraper. in a vol of .5 ml. C = Cortisone—5 mg inj. subcut. in a vol of .2 ml. P = Penicillin—10000 or 20000 units inj. subcut. in a vol of .2 ml. Sa = Saline—.2 ml inj. subcut. in a vol of .2 ml.

† No. of animals dead/total No. of animals inj.

reflected only the protective action of cortisone.

Experiments of the same type as those above were carried out using penicillin, which previously had shown no influence on the mortality rate, in place of streptomycin. The results of 2 such experiments shown in Table III indicate that penicillin is equally, if not more, effective than streptomycin in interfering with the protective action of cortisone. It will be noted that doses of penicillin which are capable of interference when given after the cortisone are without effect when given at the same time as the hormone, as was also the case with streptomycin.

Discussion. A single relatively large dose of cortisone injected one hour before, or simultaneously with, a lethal dose of endotoxin derived from a coliform bacillus consistently protected the majority of animals from death. This protection was lost, however, when the cortisone was injected one hour after the endotoxin. These results are in essential agreement with those of other workers who have shown that cortisone is most effective when administered prior to the endotoxin(5,15), although occasional protection has been demonstrated when the hormone was injected after the endotoxin(4).

It has been known for many years that some sulfonamides and antibiotics are able to protect animals from a lethal dose of endotoxins(10-14), but the mechanism of this protection is unknown. A transient bacteremia, probably of intestinal origin, occurs in some

animals following the injection of coliform endotoxin and it was postulated that antimicrobial agents might protect by suppression of the bacteremia. Our observations on the effects of chlortetracycline, penicillin, chloramphenicol, and streptomycin on the endotoxin mortality rate revealed occasional though irregular protection only by streptomycin. Other experiments have shown that the bacteremia varies considerably in degree from one animal to the next and probably does not contribute significantly to the death of the animal. In spite of the inconsistent protection obtained with streptomycin it was thought worthwhile to investigate the possibility that the combined administration of both cortisone and streptomycin might protect more of the animals from death than with the use of cortisone alone. However, just the opposite results were obtained. The injection of even small doses of streptomycin into animals previously treated with cortisone resulted in significantly higher death rates than in those animals given cortisone alone. This interference of streptomycin with cortisone protection can hardly be a specific reaction, since penicillin, a completely unrelated compound, shows the same interfering capacity. This effect likewise is probably unrelated to any antimicrobial action of these drugs. Both antibiotics were capable of interfering only when given after, and not simultaneously with, the cortisone. In view of the dearth of information on how cortisone may protect animals from the harmful effects of toxic substances(16), or on the precise mode of action of endotoxins *in vivo*(17) or on the pharmacologic effects of antibiotics below the toxic level(18), it is not profitable at this time to speculate on the reasons why antibiotics may interfere with cortisone protection against endotoxins. However, since such interference has been demonstrated with 2 completely unrelated agents, it is suggested that the interference is a nonspecific reaction and may simply involve an additional stress or "insult" to an animal already in a precarious balance. Studies on the effects of other agents alone and with cortisone on endotoxin mortality rates are currently in progress.

Summary. 1. Administration of cortisone

prior to or simultaneously with a lethal dose of endotoxin consistently protected the majority of mice from death. There was no protection when cortisone was administered after endotoxin. 2. A transient bacteremia, presumably of intestinal origin, occurred in some animals a few hours after injection of endotoxin, but probably did not contribute significantly to death of the animals. 3. Antibiotics were capable of suppressing bacteremia, but with the exception of streptomycin, which irregularly gave some protection, did not influence mortality rate. 4. Injection of streptomycin or penicillin into animals previously treated with cortisone resulted in significantly higher mortality rates than in animals given cortisone alone. This interference by antibiotics with cortisone protection was demonstrable only when antibiotics were given after cortisone; simultaneous administration of both cortisone and antibiotic gave results which reflected only protective effects of cortisone. 5. In view of the unrelated nature of the antibiotics capable of interfering with cortisone protection against endotoxins, it was suggested that the interference was probably of a nonspecific nature.

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Antagonism of Inotropic Responses of Frog Heart to Epinephrine and Acetylcholine.* (21212)

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In a previous study the ratio between the concentrations of epinephrine and acetylcholine present simultaneously was shown to be important in determining the amplitude of contraction of the isolated frog heart(1,2). With a mixture of appropriate concentrations of these drugs a response was obtained whose

amplitude was similar to that of the heart before the addition of the mediators. This latter condition was referred to as a balanced response and was characterized by the ratio of epinephrine to acetylcholine necessary to produce it. This balanced response was readily altered by the barbiturates, which decreased the response of the heart to epinephrine.

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In this investigation, using the approach

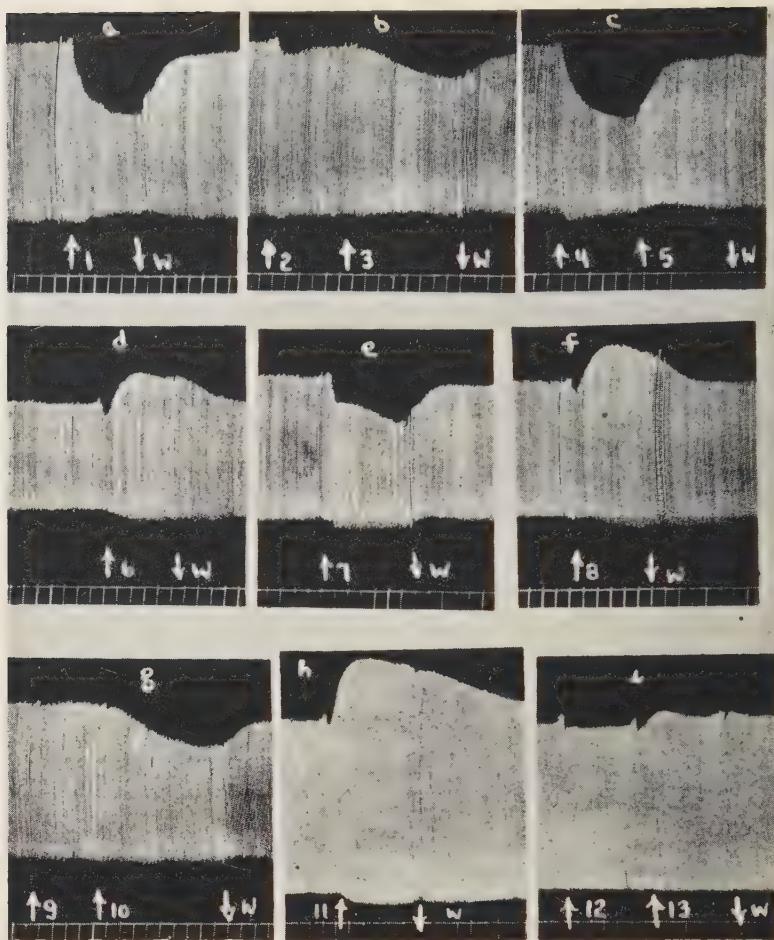


FIG. 1. Effect of atropine on inotropic response of frog heart to epinephrine (Epi) and acetylcholine (Ach).

- | | |
|---|-------------------------------------|
| 1. Ach 6×10^{-8} M | 8. Same as 7 + atropine 10^{-7} M |
| 2. " + atropine 10^{-7} M | 9. Atropine 10^{-7} |
| 3. " | 10. Ach 6×10^{-8} M |
| 4. " | 11. Epi 15×10^{-8} M |
| 5. " + <i>idem</i> | 12. Atropine 10^{-7} M |
| 6. Epi 15×10^{-8} + Ach 3×10^{-8} M | 13. Epi 15×10^{-8} M + |
| 7. " + 6 " (2.5:1) | atropine 10^{-7} M |

Arrows indicate duration of drug administration. Time in 20 sec. intervals.

described for the barbiturates(1), we studied the effect of some drugs which are known to modify the action of either epinephrine or acetylcholine. It was found that the balanced response was upset by some of these drugs, and that the same agents interfered with the inotropic actions of both epinephrine and acetylcholine under slightly different conditions.

Method. The isolated frog heart was perfused with aerated Clark's Frog Ringer solution at room temperature. At the beginning

of each experiment the responses to selected doses of epinephrine and acetylcholine were recorded. Only those hearts which showed an adequate response to both agents were used for further investigation. This criterion was used to eliminate preparations which had been either injured during the removal of the heart or were already contracting maximally and could show no further increase in amplitude. The following drugs were prepared in the Clark's solution prior to perfusion: acetyl-

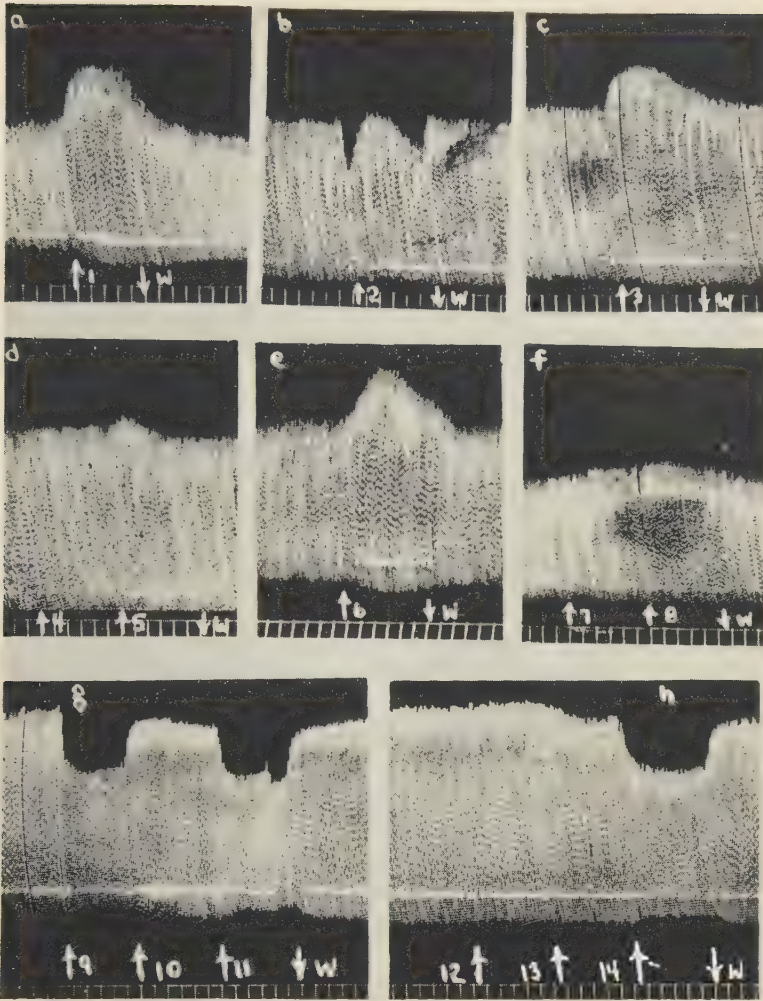


FIG. 2. Action of hexamethonium (Hex) on inotropic response to epinephrine (Epi) and acetylcholine (Ach).

- | | |
|---|---|
| 1. Epi $15 \times 10^{-8}M$ | 8. Epi $15 \times 10^{-8}M$ + Hex $5 \times 10^{-4}M$ |
| 2. " + Ach $6 \times 10^{-8}M$ (2.5:1) | 9. Ach $6 \times 10^{-8}M$ |
| 3. <i>Idem</i> + Hex $5 \times 10^{-4}M$ | 10. " + " |
| 4. Hex $5 \times 10^{-4}M$ | 11. " + " |
| 5. Same as 3. | 12. Hex $5 \times 10^{-4}M$ |
| 6. Epi $15 \times 10^{-8}M$ + Hex $5 \times 10^{-4}M$ | 13. Ach $6 \times 10^{-8}M$ + " |
| 7. Hex $5 \times 10^{-4}M$ | 14. Ach $6 \times 10^{-8}M$ |

choline chloride, l-epinephrine bitartrate,[†] atropine sulfate, hexamethonium bromide, tetraethylammonium chloride, and diethylaminoethanol. When a combination of drugs was employed, all were incorporated in the same solution. In some experiments the heart was previously perfused with a drug

[†] Furnished through courtesy of Dr. M. L. Tainter of the Sterling-Winthrop Institute.

before testing the response to epinephrine and acetylcholine. This was accomplished by first introducing the drug for a two minute period and then replacing this solution by a mixture containing epinephrine and acetylcholine as well as the drug. All of the drugs were used in concentrations which produced no changes in heart rate. The heart activity was recorded continuously with an end writing lever on a smoked drum.

manner as to permit the positive inotropic action of the epinephrine component. Prior perfusion with hexamethonium, however, blocked this latter action (†4, †5). Previous perfusion with hexamethonium also interfered with the action of epinephrine when the acetylcholine was omitted (†7, †8). At a higher concentration than that used with atropine, 5×10^{-4} , hexamethonium simultaneously perfused with acetylcholine reversed the action of the latter (†10) but under similar conditions did not affect the action of epinephrine (†6). Initial perfusion with hexamethonium, however, had no effect on the acetylcholine depression (†14). Results comparable to these were obtained with the same concentration of tetraethylammonium.

Diethylaminoethanol. Although not usually classified as an autonomic drug, diethylaminoethanol was included in this group of agents since its effects on the actions of epinephrine and acetylcholine have been studied by others (6). In Fig. 3 we note that the balanced response to a mixture of epinephrine and acetylcholine (2.5:1) was readily decreased by prior perfusion with diethylaminoethanol (†2, †3 and †4). Additional studies indicated that previous perfusion with diethylaminoethanol reduced the response to epinephrine (†7, †8) but had no effect on acetylcholine depression. At 10^{-6} M as well as at higher doses, diethylaminoethanol when given simultaneously with epinephrine was ineffective against the latter (†9). Likewise, it failed to block the response to acetylcholine (†6). At 10^{-6} M diethylaminoethanol had no inotropic action of its own.

Discussion. In the concentrations used in these experiments atropine, hexamethonium, and tetraethylammonium, when present simultaneously with acetylcholine, blocked the negative inotropic action of the latter. The hypothesis has been adopted by Clark(3) that atropine and tetraethylammonium unite with specific receptors on the heart cell surface and make these sites less accessible to acetylcholine. It would be reasonable to accept a similar explanation for hexamethonium because of its related quaternary ammonium structure(5). This competitive antagonism also explains how the balanced response to a

mixture of epinephrine and acetylcholine is upset by these drugs. In blocking acetylcholine depression, they permit an unopposed response to epinephrine. In a previous report it was shown that in the balanced response epinephrine and acetylcholine were acting independently of one another(2). The present results are in accord with this observation. In contrast to their effect on acetylcholine these compounds administered simultaneously with epinephrine do not block its action. Diethylaminoethanol had no effect on the action of either neurohumoral agent when administered simultaneously with them.

Prior perfusion with all of the drugs tested had no effect on acetylcholine depression, but all blocked the positive inotropic action of epinephrine. Riesser and Hergott(6) also observed the inhibitory action of diethylaminoethanol when it was perfused prior to the addition of epinephrine. The reason for the necessity of a short prior perfusion before these drugs will block the action of epinephrine is not clear. It appears unlikely that the time required for penetration of the drugs to the site of action is the sole explanation. Clark(4) found that the frog heart, because of its sponge-like character, permits rapid diffusion of drugs. It appears rather that a certain amount of time is required even after these compounds arrived at their site of action before the changes occur which make the heart less responsive to epinephrine. Increasing the concentration of epinephrine, however, will again give a maximal response. This indicates that during the short preliminary perfusion the threshold for the response to epinephrine is raised but that the heart can still respond to larger doses.

Our results also suggest that the inotropic responses of the isolated heart to the chemical mediators can be altered through at least two mechanisms. The first is specific and directly related to a competitive antagonism to acetylcholine; the second is non-specific and associated with an increased threshold for the epinephrine response. The increased threshold requires a few minutes to be effective, whereas the competitive mechanism occurs instantaneously. Atropine, hexamethonium, and tetraethylammonium influenced both mechan-

isms, whereas, diethylaminoethanol involved only the second.

Summary. In the isolated frog heart balanced responses to mixtures of epinephrine and acetylcholine were converted to positive inotropic responses by atropine, hexamethonium and tetraethylammonium. These drugs specifically and competitively antagonized acetylcholine depression when present with the latter. Prior perfusion with them as well as with diethylaminoethanol effectively blocked the positive inotropic action of epinephrine. This antagonism is believed to be non-specific and related to changes which raise the threshold to epinephrine.

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Life Maintaining Action of 9 Alpha Chlorohydrocortisone Acetate in Adrenalectomized Rats.* (21213)

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Chlorohydrocortisone acetate is 4 times more active than cortisone acetate in promoting deposition of liver glycogen(1). Furthermore, chloro-F-acetate is more active than hydrocortisone or cortisone acetate in extending survival of adrenalectomized rats given one injection(2). Since single injection experiments appraise duration of action rather than daily hormone requirements, a comparison was made of the daily requirements for chloro-F-acetate and cortisone acetate in adrenalectomized rats.

Methods. Immature male rats (Long-Evans strain) were adrenalectomized when 60-65 g. All rats were fed *ad libitum* a diet consisting of 20% casein, 22% dextrose, 25% dextrin, 25% Crisco, 2% corn oil, 2% cellulose flour and 4% Wesson's salt mixture. Each kg of diet was supplemented with 2 g choline chloride and vitamins (Table I). The hor-

mones used were chloro-F-acetate (Squibb)[†] and cortisone acetate (Merck) in aqueous suspension.

Results. Chloro-F-acetate provided 100% survival for 20 days when given to adrenalectomized rats in 15 μ g amounts daily whereas 150 μ g of cortisone acetate was required to provide similar protection (Table II). Ten μ g and 100 μ g daily of the 2 steroids proved inadequate for 100% survival of the adrenalectomized rats suggesting a 10:1 ratio of activity. Preliminary studies indicate that 15-20 μ g of desoxycorticosterone acetate[†] (DCA in oil) will provide 100% survival of adrenal-

TABLE I. Vitamin Supplements/100 g of Diet.

Alpha tocopherol	4.0 mg
Vit. A	900 U.S.P. units
" D	180 <i>idem</i>
	mg
2-methyl-1,4 naph-quinone diacetate	1.0
Thiamin HCl	.8
Riboflavin	1.6
Pyridoxine HCl	.8
Niacin	4.0
Calcium pantothenate	4.4
Para aminobenzoic acid	4.0
Inositol	21.6

* Supported by U. S. Public Health Grant A-462.

[†] Grateful acknowledgement is made to E. R. Squibb & Sons for making the chlorohydrocortisone acetate available and to Ciba Pharmaceutical Products for the desoxycorticosterone acetate (per-corten).

TABLE II. Survival of Adrenalectomized Immature Male Rats Treated with Chloro-F-Acetate or Cortisone Acetate.

Daily treatment, mg	No. of rats ADX	Rats alive after 20 days	Avg body wt gain in 20 days, g
Cortisone acetate			
.10	15	7	2
.15	10	10	13
Chloro-F-acetate			
.010	11	7	41
.015	10	10	56
.025	10	10	63
.00	20	0	—

ectomized rats on the same diet. Thus, chloro-F-acetate simulated DCA in potency.

Body weight gain exhibited by cortisone acetate-treated rats was only 13 g as compared to the 56 g body weight gain attained on chloro-F-acetate. These data emphasize the lack of correlation between survival and body weight gain.

Longevity of steroid action can be tested by the survival of rats following one injection given the day of adrenalectomy. Previous studies revealed that 1 mg of cortisone acetate had little effect on survival of adrenalectomized immature rats whereas 5 mg doubled the life span(3). For the current study, groups of 10 immature male rats were adrenalectomized and fed the purified diet. Untreated rats survived an average of 7 days whereas one subcutaneous injection of 2.5 mg of cortisone acetate extended survival to 10.5 days. Considering chloro-F-acetate as 10 times more active than cortisone acetate, a .25 mg dosage was used. Chloro-F-acetate provided an 18.2 day average survival indicating a more prolonged action than cortisone

acetate and on this basis a greater than 10:1 ratio of activity. One mg of DCA (in oil) had no effect on survival and single injections did not simulate chloro-F-acetate activity as observed with daily injections.

The influence of dietary protein on hormone action was observed in 12 adrenalectomized immature male rats. Rats receiving .25 mg of chloro-F-acetate and fed a protein-free diet survived 12.2 days; 6 days less than rats fed casein but significantly longer than untreated controls. Normal rats readily survive 20 days on the protein free diet.

Hydrocortisone will inhibit the uterine weight increasing action of estradiol(4). Preliminary studies using 1 μ g of estradiol benzoate and 2.5 mg of chloro-F-acetate revealed no antagonism.

Summary. The daily dosage requirement of 9 alpha chlorohydrocortisone acetate to permit survival of adrenalectomized rats for 20 days indicates that this compound is 10 times more active than cortisone acetate. Activity was equal to or slightly greater than desoxycorticosterone acetate. A single injection of chloro-F-acetate (.25 mg) provided a longer survival of adrenalectomized rats than cortisone acetate (2.5 mg). Hormone effectiveness was influenced by diet.

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Blood Pressure in Patients with Hypertension Following Intramuscular Chlorpromazine.* (21214)

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The effectiveness of chlorpromazine as an antiemetic agent(1,2,5) has resulted in extensive clinical trials of the drug to combat nausea and vomiting produced by drugs, irradiation and various disease states. Prior to its use in combination with antihypertensive agents, which not infrequently induce nausea and vomiting as undesirable side effects, it seemed worthwhile to evaluate the action of chlorpromazine alone on the blood pressure of hypertensive man.

Methods. Ten adults, 7 males and 3 females, with persistent benign essential hypertension were studied in the postprandial state. Fourteen separate injections of chlorpromazine in a range of dosage from 25 to 100 mg were given deep in the gluteal region. Blood pressures were measured in the arm with a mercury sphygmomanometer. The pulse rates and subjective manifestations were also recorded. Control observations on the pulse and blood pressure were made every 15 minutes for 1½ hours with the patient recumbent and during quiet standing. Similar observations were continued at 15 minute intervals for 3 hours after administration of the drug and subsequently at 30- to 60-minute intervals for an additional 3 to 7 hours.

Results. The effect of intramuscular chlorpromazine on the blood pressure, the average values for recumbent and standing blood pressures as well as the range of measurements during the control period are shown in the table. The standing blood pressure after intramuscular chlorpromazine in each instance is the lowest pressure observed during the experimental period and represents the maximal hypotensive effect. The recumbent pressure occurring concomitant with the maximal

hypotensive effect is also recorded. The most striking action was the development of postural hypotension. The lowering of the standing blood pressure was apparent within 15 to 30 minutes after chlorpromazine was given, but the maximal effect did not occur until 3 to 4 hours had elapsed. The systolic pressure usually fell to normal or subnormal level and with 4 exceptions was accompanied by a similar lowering of the diastolic pressure. Recumbent pressures showed no significant change from those recorded during the control period. Even in this small series some variation in dose response is apparent.

All the patients experienced drowsiness, usually mild, and particularly with a dose of 50 mg or more. Patients 2, 3, 4, 7 and 8 complained of dizziness on standing at the time of the maximal hypotensive effect. In no instance did syncope occur and the dizziness was promptly relieved by resuming the recumbent position. In patients 3 and 4 the complaint of dizziness was relieved by mild exercise (slow walking), although this produced no remarkable change in the standing blood pressure or pulse rate. Compensatory tachycardia during standing was not a prominent feature and consisted of an increase in the pulse rate of 10 to 40 per minute. The blood pressure changes in patient 5 are presented in Fig. 1 to illustrate the typical response to chlorpromazine.

Discussion. To our knowledge, this is the first study demonstrating a consistent orthostatic fall in blood pressure following intramuscular administration of chlorpromazine to hypertensives. Previous investigations(3-5) have shown only a negligible effect of average doses on the blood pressure of the anesthetized dog and an occasional episode of transient postural hypotension in man following intramuscular administration of large doses. Two recent reports(6,7) describe the use of chlorpromazine in psychiatric disorders and men-

* Generously supplied by the Smith, Kline and French Laboratories. "Chlorpromazine is the generic name for SKF 2601-A, RP 4560, 'Largactil', 'Mega-phen', 'Amplactil', and the U. S. Trade Name 'Thorazine'."

TABLE I. Effect of Intramuscular Chlorpromazine on Blood Pressure of 10 Hypertensives.

Dose, mg	Blood pressure				Minimal post-inj.	
	Pre-injection				Recumbent*	Standing
	Avg	Range	Avg	Range		
100	172/113	165/110-190/120	152/110	145/110-162/110	170/120	125/105
75	187/120	185/120-190/120	185/122	175/120-180/125	170/120	125/110
50	169/108	158/102-180/120	165/116	158/114-178/120	170/118	100/ 68
50	210/106	194/100-222/108	196/110	188/104-200/115	160/100	80/ 50
50	213/111	200/100-220/118	188/118	162/118-218/120	210/110	100/ 60
25	239/125	232/120-246/128	210/128	204/128-212/130	210/120	130/ 80
50	232/124	225/120-240/140	214/136	210/120-230/140	202/108	112/ 80
50	231/143	220/140-246/150	212/152	210/150-228/155	220/130	100/ 80
25	232/116	222/110-242/130	217/127	210/126-236/130	228/128	176/120
50	224/118	214/118-234/118	203/125	190/120-230/130	190/116	142/103
25	233/130	230/128-240/130	197/123	192/120-200/128	164/ 98	118/ 80
25	190/103	180/ 92-200/120	167/112	148/ 98-180/120	160/ 94	128/ 90
50	190/110	182/110-202/110	172/117	158/108-192/130	144/ 98	90/ 70
50	196/106	195/100-200/120	174/100	162/ 94-180/116	188/ 98	96/ 76

* Immediately preceding lowest standing B. P.

tion postural hypotension as a prominent side effect following both oral and intramuscular administration of the drug, but no data on changes in blood pressure are presented.

The absence of a significant change in the recumbent blood pressure implies that the principal action of the drug is through the blockade of barostatic reflexes which normally sustain the blood pressure during standing. Pharmacological investigations of the cardiovascular actions of chlorpromazine have re-

vealed that the drug has adrenolytic effects. Courvoisier *et al.*(3), who demonstrated an antagonistic action of chlorpromazine to the pressor effects of epinephrine and nor-epinephrine infusions, also found that the drug abolished the pressor response to carotid occlusion and central vagus stimulation. The adrenolytic action of chlorpromazine has been confirmed by Finkelstein *et al.*(8) and Melville (9). It should be emphasized, however, that the mechanism of action of the drug on blood pressure remains poorly defined.

The postural effect of chlorpromazine in hypertensives may have clinical implications. Its effect on the blood pressure is similar to that of ganglionic blocking agents such as hexamethonium and it produces little or no change on the blood pressure during recumbency. The drug may be of value in combination with other antihypertensive agents whereby its hypotensive and antiemetic properties may be utilized to advantage.

Summary. Chlorpromazine, in an average dose of 50 mg, was administered by the intramuscular route to 10 human hypertensives. The drug was found to produce a significant decrease in blood pressure during standing, and in some instances, postural hypotension. Blood pressure in the recumbent position showed no significant change.

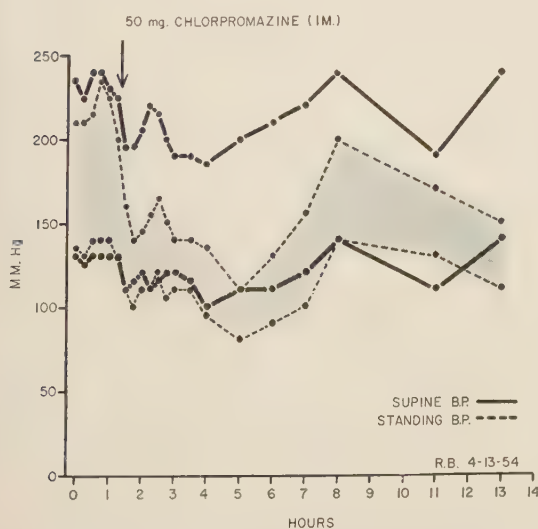


FIG. 1. Action of chlorpromazine on blood pressure of a 45-year-old colored male with hypertension. Note fall in systolic and diastolic pressures in standing position with inconsistent changes in recumbent pressures.

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Conjugated Steroids V. Hydrolysis of Total Ketosteroids in Urine.* (21215)

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Numerous attempts have been made to effect a complete nondestructive hydrolysis of ketosteroids without affecting in any way the nature of the steroids. It has been possible to hydrolyze the ketosteroid glucuronides under mild conditions with enzymes prepared from bacteria(1) or spleen(2). Recently, Cohen and Oneson have described the hydrolysis of ketosteroid sulfates with dioxane-trichloroacetic acid(3). About 40% of the total ketosteroids of normal male urine are apparently conjugated as sulfates, of which slightly more than half is of the beta configuration. The sum of the ketosteroid sulfates and glucuronides exceeds the value obtained by routine hydrochloric acid hydrolysis by about 15%. This paper describes the hydrolysis of the total ketosteroids by the use of dioxane-HCl.

Methods. Urine residues are prepared in the same manner as for the hydrolysis of the ketosteroid sulfates(3). This involves extraction with butyl alcohol of acidified (pH 2-3) urine. The extract is washed with water and evaporated to dryness at temperatures not exceeding 60°. Portions of the butyl alcohol residues equivalent to 100 to 200 cc of urine are hydrolyzed by the addition of 10 cc of 1,4 dioxane containing 10% of concentrated

hydrochloric acid. The alpha and beta fractions are assayed after their separation with digitonin according to the method of Frame (4).

Results. A concentration of acid which

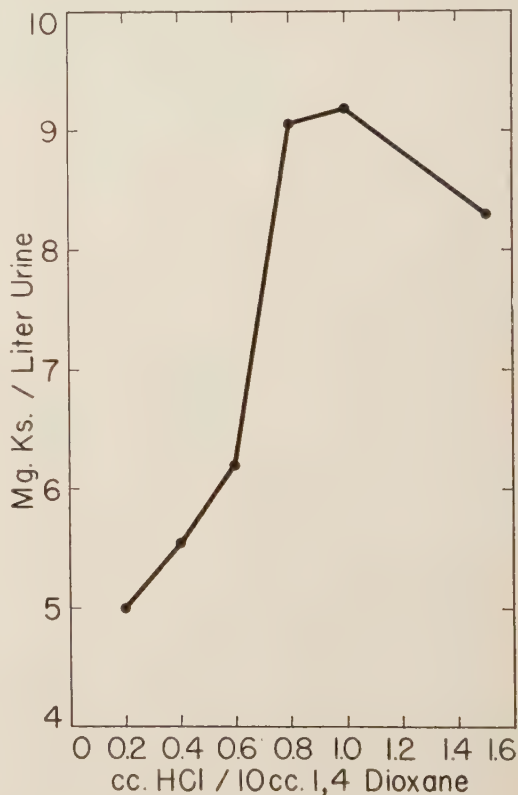


FIG. 1. Release of ketosteroids by dioxane HCl.

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TABLE I. Standard HCl vs Dioxane, Dioxane-HCl and Glucuronidase.

Urine batch	mg Ks/l urine								
	Standard HCl total	(a) Dioxane total	α	β	(b) Glucuronidase total	a + b	Dioxane-HCl		
							Total	α	β
NMU 24	10.2	2.5	1.3	1.2	8.2	10.7	12.0	10.3	.94
27	9.2	3.8	1.5	2.0	7.4	11.2	10.4	8.4	1.9
124	9.2	3.8	1.4	2.3	5.8	9.6	9.5	7.1	2.4
153	10.2	4.2	2.2	1.7	6.8	11.0	11.7	10.2	1.8
168	10.7	4.6	2.2	2.4	7.2	11.6	12.2	9.9	2.2
215A	11.0	4.4	2.1	2.2	7.4	11.8	13.5	9.8	3.0
NMU 216	9.8	4.4	1.3	2.8	7.5	11.9	13.3	10.7	2.4
LPU 20	5.5	2.2	1.6	.6	3.4	5.6	6.1	5.1	.7

effects maximum hydrolysis was found by dissolving the residue in 10 cc of 1,4 dioxane; after adding 0.1-1.4 cc (1-14%) concentrated HCl, the preparation was heated in a steam bath for 10 minutes (see Fig. 1). It can be seen that 8-10% HCl gives a maximum hydrolysis of the urinary ketosteroids. Results of the dioxane-HCl hydrolysis on various urine residues are shown in Table I. It can be seen from these data that about 17% more steroids are formed than by the standard HCl procedure. About 20% of the total steroids were found to be of beta type. Heating with an excess of acid or heating for a long period of time results in the destruction of the beta steroids. It is important to point out that longer heating causes destruction of the ketosteroids. Thus, Urine NMU 153 heated with

dioxane HCl for 10 minutes resulted in the release of 2.1 mg of beta steroids. After heating for 15 minutes, the beta steroids fell to 1.97 mg, and after heating for one hour the beta steroids were 0.94 mg.

Summary. The total ketosteroids of urine may be readily hydrolyzed by dioxane-hydrochloric acid. This hydrolysate contains about 20% of the total steroids as beta type.

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Increased Metabolic Rate without Thyroid Participation on Injection of Rats with Pituitary Erythropoietic Fractions.* (21216)

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As reported recently, extracts from sheep pituitary can be obtained(1) containing an erythropoietic principle which repairs the anemia resulting from hypophysectomy(1-3), prevents the development of the neonatal anemia(4,5), and produces polycythemia in the normal(6), hypophysectomized(7,8), and adrenalectomized rat(2). Reticulocytes and normoblasts appear in the peripheral blood of

hypophysectomized rats injected with this erythropoietic factor, and their bone marrow is stimulated. Inasmuch as reports have appeared in the literature indicating that increased activity of the bone marrow is accompanied by an elevated metabolic rate(9-11), the caloric output of rats injected with the pituitary erythropoietic fraction was examined.

Methods. Female rats of the Long-Evans

* Supported in part by U. S. Public Health Grant.

strain were hypophysectomized when 26-28 days old and were maintained for 45 days.[†] During this postoperative period the red cell volume decreased to a level half that of normal(6). Injections of pituitary erythropoietic preparations(1) were subsequently given daily for a period of 14 days. The substances were administered subcutaneously in a beeswax and oil mixture. The red cell volumes were determined at the end of the experimental period, using the Fe⁵⁹ labeled cell method(12), in order to ascertain the effectiveness of these erythropoietic fractions in repair of the post-hypophysectomy anemia. Histological examinations of the target organs of the pituitary were made in order to determine the presence in the injected material of contamination by known pituitary hormones. Completeness of hypophysectomy was confirmed at autopsy by examination of the sella turcica, and no data have been included in the tables except from animals completely hypophysectomized. The basal metabolic rate was determined two days before the termination of the experiment. A fast of 10 hours preceded this test.[‡]

Results. All of the pituitary preparations listed in Table I stimulated erythropoiesis and repaired the post-hypophysectomy anemia. Increased calorigenesis also characterized all groups. The caloric output increased 78 to 101% so that the metabolic rate returned from the hypophysectomized level to the normal.

[†] All rats used were injected intraperitoneally for 2 consecutive days after hypophysectomy with 0.5 cc of 0.5% Terramycin in 5% glucose. The injections were repeated once every 10 days for the period of the experiments. Rats with respiratory infections were excluded from the experimental groups. The rats were maintained on a standard laboratory diet (Diet XIV) given *ad libitum* and kept in a constant temperature room throughout the experiment (75 ± 2°F). The standard laboratory diet, given dry, was supplemented every afternoon by a wet mash of Diet I. Lettuce was given 2 to 3 times weekly. Diet XIV consists of 68.5% wheat, 5% casein, 10% fish meal, 10% alfalfa leaf meal, 5% fish oil, 1.5% NaCl, KI added (analysis 1 µg iodine per g diet). Diet I consists of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.0% fish oil, 0.75% NaCl, 1.5% CaCO₃, KI added (analysis 1 µg iodine per g diet).

As the pituitary preparations which stimulated erythropoiesis also increased calorigenesis, the possibility of thyrotrophic hormone contamination in the preparations was considered. Histological examination of the thyroids showed evidence of minimal activity in thyroids of some groups, but no stimulation in others (Table I).[§] The increased metabolic rate was of the same order in all groups, despite variability in thyroid histology.

The functional capacity of thyroids of the rats treated with the pituitary erythropoietic factor, as judged by their ability to concentrate tracer doses of I¹³¹, paralleled these histological findings.

In order to apply this test of function, red cell volume determinations were first made by withdrawal of blood from the external jugular. A small dose of I¹³¹ (1 µc) was then injected intraperitoneally and the thyroids were removed 24 hours later, fixed in Bouin's fluid, and the radioactivity was counted directly in a scintillation counter(14).

The I¹³¹ uptake of the thyroids in groups showing histological evidence of thyroid stimulation was 2.1%, in contrast to 0.9% in the hypophysectomized controls. This increase in I¹³¹ uptake was, however, only a fraction of

[‡] Determination of oxygen consumption was performed by the method and with the apparatus described by Kleiber(13). Surface area was calculated

by the formula: $m^2 = \frac{9 \sqrt{(BW)^2}}{10,000}$. We wish to thank

Dr. Arthur H. Smith, University of California, Davis, for his cooperation in confirming our determinations of oxygen consumption.

[§] Thyroid activity is designated in the tables as follows: + Definite, though low, stimulation, the epithelium is low and the cytoplasm scanty; nuclei are slightly rounded and chromatin particles are more easily distinguishable than in hypophysectomized controls, and the hematoxylin stain is somewhat paler; the colloid stains a lighter eosin tint.

++ The epithelium is low cuboidal; nuclei are vesicular and light staining; there are some vacuoles in the colloid.

+++ The thyroid vesicles are smaller and epithelium is higher cuboidal; there is marked vacuolation of the colloid.

++++ Almost complete resorption of colloid has occurred.

TABLE I. Increased Calorigenesis in Hypophysectomized Rats during Administration of Pituitary Erythropoietic Fractions.

Preparations inj.	Dose, mg	No. of rats	Metabolic rate		Red cell vol		Thyroid† histology	% I ¹³¹ uptake
			Cal/m ² /hr	% of normal	ml/100 g BW	% of normal		
II-H-114 (B)	.10	8	48.0 ± 1.7*	109	2.65 ± .10	115	+(3), ++(5)	2.1 ± .2
122 (B)	.05	7	42.6 ± 1.3	97	2.67 ± .11	116	+(6), —(2)	2.0 ± .2
112 (2)	.05	7	44.3 ± 3.0	100	2.51 ± .02	109	+(3), —(5)	1.0 ± .1
107	.10	8	45.9 ± 1.8	104	2.49 ± .11	108	+(2), —(6)	.9 ± .1
H controls	—	45	23.9 ± 1.4	54	1.50 ± .05	65	—	.9 ± .1
N "	—	15	44.0 ± 1.0	100	2.30 ± .05	100	—	7.5 ± .3

† Degree of thyroid stimulation indicated is defined in text footnote. No. of rats is given in parentheses.

$$* SE = \frac{\sqrt{\sum d^2}}{\sqrt{n(n-1)}}$$

the normal uptake (7.5%). The I¹³¹ uptake was not increased in any thyroids which did not subsequently show histological evidence of stimulation.

The small contamination of thyrotrophic hormone present in some of the erythropoietic preparations[†] did not adequately explain the increased calorigenesis. However, since some degree of thyroid stimulation was caused by erythropoietically active preparations, a comparison was made between the calorigenic action of these preparations and that of the most potent thyrotrophic preparations available.[‡] Groups of hypophysectomized rats were injected intraperitoneally, daily for 14 days, with doses of thyrotrophic hormone in saline ranging from 5 to 450 μ g. The metabolic rates were determined after the injection had been given for 12 days. Red cell volume determinations were made at the end of the 14-day period, the blood for the determination being withdrawn from the external jugular without sacrificing the rats. The ability of the thyroid to concentrate I¹³¹ was determined by injecting a tracer dose of I¹³¹ (1 μ c) immediately after determination of the red cell volume. Twenty-four hours after radio-iodine adminis-

tration the rats were sacrificed, the thyroids were fixed in Bouin's fluid, and the radioactivity was counted in a scintillation counter. The histology of the thyroids was later examined. Metabolic rates and red cell volumes were likewise determined for intact rats of the same age, in order to have normal values for comparison.

No significant erythropoietic response occurred in groups receiving the lower levels (5 to 150 μ g) of thyrotrophic hormone (Table II). At the higher levels (300 to 450 μ g), there was only a 30% increase in red cell volume. This increase is the same as that which resulted from injection of 2.0 to 3.5 μ g daily of thyroxine(6), a dose judged to be physiological in that it had been found adequate for normal growth and skeletal differentiation in the hypophysectomized rat(15).

The calorigenic output of the hypophysectomized rats (Table II) was not brought back to normal levels by even the highest doses of thyrotrophic hormone given, doses which were adequate to cause an increase in the height of epithelial cells, increased size of nuclei, almost complete colloid resorption and almost normal I¹³¹ uptake. Thus, thyrotrophic hormone, at doses more than adequate to repair thyroid morphology and reinstate normal I¹³¹ uptake, failed to increase the caloric output to the normal level. This result supported the concept that the metabolic activity of erythropoietically active pituitary preparations did not stem from their content of thyrotrophic hormone.

Increased calorigenesis resulting from injec-

[†] None of the preparations contained recognizable amounts of FSH, ICSH or growth hormone at the doses injected over the 14-day period.

[‡] The purified thyrotrophic hormone used had been fractionated from beef anterior pituitary and its potency standardized in hypophysectomized rats by the histological response of the thyroids and by I¹³¹ uptake. By both of these criteria the preparation was effective at the minimal dose of 5-10 μ g(15).

TABLE II. Calorigenic Response of Hypophysectomized Rats to Graded Doses of Pituitary Thyrotrophic Hormone.

Group	Dose of TSH, mg	No. of rats	Metabolic rate		Red cell vol		Thyroid histology	% I ¹³¹ uptake
			Cal/m ² /hr	% of normal	ml/100 g BW	% of normal		
\bar{H} inj.	.005	6	22.1 \pm .8*	50	1.52 \pm .05	66	\pm	—
	.010	5	25.1 \pm .9	57	1.51 \pm .06	66	+	—
	.050	6	27.0 \pm 1.3	61	1.59 \pm .04	69	++	—
	.150	6	27.4 \pm .9	62	1.73 \pm .08	75	+++	4.5 \pm .5
	.300	6	29.0 \pm .8	66	1.96 \pm .10	85	++++	6.0 \pm .8
	.450	6	30.5 \pm 1.2	69	1.95 \pm .10	85	++++	6.9 \pm .5
\bar{H} controls	—	45	23.9 \pm 1.4	54	1.50 \pm .05	65	—	.9 \pm .1
N "	—	15	44.0 \pm 1.0	100	2.30 \pm .05	100	—	7.5 \pm .3

$$* SE = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

tion of the erythropoietic factor in the absence of the thyroids would furnish conclusive evidence that the change in oxygen consumptions did not mediate through the thyroid. In order to test this point the thyroid tissue was destroyed by the administration of I¹³¹. A dose of 1 mc was given at 28 days of age and followed 15 days later by a second dose of 500 μ c. After a lapse of 4 months, those animals were selected in which destruction of the thyroids was most probably complete. The choice was made on the basis of poor muscle tone, persistence of juvenile hair, body growth stasis, and low metabolic rate. From a group of 30 rats, 16 were selected in which the oxygen consumption was 40% or more below normal. Half of this group was injected for 14 days daily with 100 μ g of an erythropoietically active pituitary preparation (II-H-109-3). Red cell volumes and metabolic rates were determined at 2 and also at 3 weeks after the onset of injection. The remainder of the rats, in which it was judged thyroid destruction was complete, were retained as controls and red cell volumes were determined at the same intervals as in the injected animals.

The completeness of thyroid aplasia in the rats chosen for the experiment was further checked terminally by the administration of a tracer dose of I¹³¹ (1 μ c) 24 hours before autopsy. The thyroid region was dissected, placed in Bouin's fluid in a vial, and the I¹³¹ uptake in the thyroid region was counted directly in a scintillation counter(6,14). Only those animals judged completely free of func-

tional thyroid tissue, by inability to concentrate radio-iodine, were included in presenting the results in Table III.

A progressive increase in the metabolic rate occurred in the injected thyroidectomized animals during the 3-week period of injections of the pituitary erythropoietic factor (Table III). After 21 days, the increase was 36% and the metabolic rate was 87% of normal. Uninjected controls showed no change in metabolic rate during this period. The erythropoietic preparations, therefore, caused a significant increase in the metabolic rate in the absence of functional thyroid tissue. The red cell volume of the thyroidectomized rats was increased to the normal level by the dose of erythropoietic principle administered. The increase in metabolic rate of hypophysectomized rats injected with pituitary erythropoietic fractions without thyroid stimulation is difficult to interpret. It would appear that the increase of calorigenesis is not mediated through the thyroid as increased caloric output occurred in the hypophysectomized rat without stimulation of the thyroid gland, and also in the absence of functional thyroid tissue. Furthermore, increased thyroid activity, judged by morphology and iodine uptake, occasioned by large doses of thyrotrophic hormone, was accompanied by only a small increase in metabolic rate. As the erythropoietically active hormone has not been prepared in pure form, it cannot be said at present whether the increased metabolic rate, in the absence of the thyroid, is due to the same or

TABLE III. Increased Calorigenesis during Administration of a Pituitary Erythropoietic Fraction* in Rats Deprived of Functional Thyroid Tissue.

Group	Days after onset	No. of rats	Metabolic rate		Red cell vol		Terminal I ¹³¹ uptake (5) thyroid region
			Cal/m ² /hr	% of normal	mg/100 g BW	% of normal	
Inj.	0	6	25.2 ± 1.3†	57	1.87 ± .05	81	—
	14	5	34.2 ± 1.8	78	2.18 ± .03	95	—
	21	5	38.2 ± 1.9	87	2.25 ± .03	98	.003
Uninj.	0	6	24.8 ± 1.3	56	1.84 ± .05	80	—
	14	6	26.0 ± .1	59	1.84 ± .05	80	—
	21	6	25.5 ± .5	58	1.80 ± .05	78	.003
Controls	21	15	44.0 ± 1.0	100	2.30 ± .05	100	7.5 ± .3

* 100 µg daily for 21 days of Preparation II-H-109-3.

$$\dagger SE = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

a different substance from that which stimulates erythropoiesis.

Clinical observations have supported the concept that metabolic rate does not depend entirely on the status of thyroid function. A high basal metabolic rate is usually observed in leukemic patients, whereas a normal I¹³¹ uptake by the thyroid of such patients has been noted(17). On the other hand, in the so-called hypo-metabolic state, where the basal metabolic rate is subnormal, there is no other evidence of decreased thyroid function (18).

Summary. 1. Erythropoietically active pituitary preparations increased the metabolic rate of hypophysectomized rats to normal levels with little or no stimulation of the thyroids. 2. A potent thyrotrophic preparation was less effective in increasing oxygen consumption than the erythropoietic factor. 3. The erythropoietic factor increased the caloric output to normal levels in rats in which all functional thyroid tissue had been destroyed.

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Transmission of Ak Leukemic Agent into Newborn Mice of the C57 Brown/cd Inbred Line.* (21217)

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In previous experiments, cell suspensions (1,2) or centrifuged or filtered (3-7), extracts prepared from livers, spleens, and lymphatic tumors of leukemic Ak (subline Ak-n) mice, were inoculated into newborn mice of the C3H inbred line. These Ak leukemic cells multiplied rapidly, causing leukemia within 2 to 4 weeks, but did not change their genetic specificity, since they could be readily grafted back to the Ak donors, but not to adult hosts of the recipient C3H line (5). On the other hand, when either centrifuged or filtered Ak leukemic extracts were inoculated into newborn C3H mice, leukemia, which developed in some of the inoculated animals after a considerable interval of time ($3\frac{1}{2}$ to 27 months), was specific for recipient strain, and could be readily transplanted, by cell transfer, to adult mice of recipient C3H line, but only exceptionally to the donor Ak strain. These results suggested that when either centrifuged or filtered Ak leukemic extracts were used for inoculation of newborn C3H mice, the resulting leukemia was caused by a cell-free, transmissible agent, acting on cells of its new host.

The purpose of this report was to determine whether mice of another low-leukemic inbred line could also be used for inoculation with Ak leukemic cells, and particularly with either centrifuged or filtered Ak leukemic extracts. The use of a different recipient strain for inoculation of Ak leukemic extracts appeared indicated because among C3H mice that had been inoculated with either filtered (6,7), or centrifuged and then diluted (8), Ak leukemic extracts, some developed typical leukemia, others, however, unexpectedly developed salivary (parotid) gland carcinomas. Without discussing the possible origin of these salivary gland tumors, it appeared of interest to determine whether another strain of recipient mice might also prove susceptible, under similar ex-

perimental conditions, to development of either leukemia, or salivary gland carcinomas. For that purpose, mice of the inbred strain C57 brown, subline cd, were used for inoculations.

Materials and methods. Leukemic extracts. Six- to 11-months-old Ak (subline Ak-n) female mice that developed leukemia spontaneously, or, in a few instances, young adult (2- to 3-months old) Ak males and females that developed leukemia as a result of implantation of Ak leukemic cells, were used as donors of the leukemic organs. Under ether anesthesia, livers, spleens, mesenteric tumors and peripheral lymph glands were removed aseptically, and ground with sterile, physiological sodium chloride solution, at 0°C, to obtain suspensions of 20% concentration. These cell suspensions were used immediately for inoculation. In the series in which centrifuged extracts were used, the leukemic cell suspensions, prepared as described above, were centrifuged at 3000 rpm (1400 x g) for 15 minutes at 0°C; the supernatant was removed and centrifuged at 9500 rpm (7000 x g) at 0°C for 10 minutes. The final supernate was then placed in glass tubes chilled to 0°C. This supernate was passed through Selas (porosity 02 or 03) microporous porcelain filter candles, under vacuum pressure of 20 to 25 mm of mercury. Each filter was tested, before and after filtration, and found to be impervious to *E. coli*. (In 2 experiments, the centrifuged leukemic extracts (15 cc) were mixed with one cc of a 1:500 dilution of fresh broth culture of *E. coli*, and then passed through Selas filter candles. The filters retained the *E. coli*, since the filtrates were sterile.) The filtered extracts were immediately placed in sterile glass tubes at 0°C. The tubes containing centrifuged, or filtered, leukemic extracts were immersed in larger glass containers filled with ice cubes and water, and kept in refrig-

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erators. The extracts were thus kept at 0°C for periods not exceeding 24 hours, prior to inoculation. All extracts were ice-cold when injected. The inoculations were subcutaneous, approximately 0.1 cc injected into each newborn mouse. In most experiments, leukemic extracts heated in electric water bath at 67°C to 80°C for 30 to 60 minutes, were inoculated into litter-mate infant mice; in one experiment 3 litter-mates were left untreated.

Animals. Mice of the low-mammary-tumor inbred strain C57 brown, subline cd (C57BR/cd) were used as test animals for inoculations of the Ak leukemic extracts, because this inbred line is essentially free from spontaneous leukemia. According to information from the Jackson Memorial Laboratory(9), about 4% of very old animals of this line have been observed to develop spontaneous neoplasms of reticular tissue, usually lymphosarcomas. Six couples of 78th and 79th generations of C57BR/cd inbred line of mice were obtained in 1952 through the courtesy of Dr. E. Russell of the R. B. Jackson Memorial Laboratory, Bar Harbor, Me., and from this nucleus a small colony raised in this laboratory by brother-to-sister mating. Within a few months a sufficient number of descendant mice became available so that couples of brothers and sisters could be placed in individual cages. Mice born to these couples in successive litters were used for inoculations of leukemic extracts. Additional young adult C57BR/cd mice obtained from the Jackson Memorial Laboratory in 1952 and 1953, were not used for breeding, but only for those experiments which dealt with the transplantation of leukemic cells. We have observed neither spontaneous leukemia nor any other tumors among our untreated mice of the C57BR/cd inbred line. Fifty-three untreated mice died when 6 to 13 months old, without leukemia or tumors. Forty-seven untreated males and females (7 to 20 months old) are now in good health.

Experimental. Results of inoculation of Ak leukemic cells. Newborn C57BR/cd mice were susceptible to implanted Ak leukemic cells. Of 33 mice, inoculated when less than 2 days old, 29 developed subcutaneous tumors at the site of inoculation, followed by generalized leukemia, in most instances within 3 to 4

TABLE I. Results of Inoculation of Ak Leukemic Cell Suspensions into C57BR/cd Infant Mice.

Route of of inoc.	Dose, cc	Age at inoc., days	No. of mice inoc.	No. of mice devel. leuk.	Incub. time, days
s.c.	.1-.2	<1-<2	33	29	15-63
s.c. or i.p.	.15-.2	4-12	34	8	15-49
"	.15-.5	14	27	6	15
"	.15-.5	20-24	17	0*	

* In 2 mice small subcut. tumors developed at site of inoculation within 2 weeks, but later regressed spontaneously.

weeks. Of 34 suckling infant mice inoculated when 4 to 12 days old, only 8 developed leukemia. Of 27 suckling infants inoculated when 14 days old, 6 developed leukemia. When 20- to 24-day-old mice were inoculated, no leukemia resulted (Table I), but 2 of the 17 inoculated mice developed subcutaneous tumors within 2 weeks at site of inoculation; these tumors persisted for several days, then regressed completely. When 53 adult (2 to 11 months old) males and females were inoculated either intraperitoneally (41 mice) or subcutaneously (12 mice) with large doses (0.5 to 5 cc) of Ak leukemic cell suspensions, none developed leukemia.

Further transplantation experiments. In 4 experiments, the leukemic tumors in suckling mice following implantation of Ak leukemic cells, were used for preparation of cell suspensions, which were then implanted intraperitoneally into adult mice (2 to 4 months old) of the donor Ak strain (14 mice) and into those of the recipient C57BR/cd inbred line (14 mice). The 14 Ak mice developed leukemia within 2 to 3 weeks following inoculation, whereas the 14 C57BR/cd mice remained in good health. These results indicated that the Ak leukemic cells, which had been implanted into suckling C57BR/cd mice, retained their genetic Ak properties, even though growing and multiplying in a foreign (C57BR/cd) host. Accordingly, they were readily transplanted to adult mice of the donor Ak strain, but could not be transferred, by cell inoculation, into adult hosts of the recipient C57BR/cd inbred line(5).

Inoculation of centrifuged or filtered Ak leukemic extracts into newborn C57BR/cd mice. The centrifuged (7000 x g) Ak leu-

kemic extracts were inoculated into 71 newborn C57BR/cd infant mice in 16 experiments, average age 6 hours.[†] Of the 71 inoculated mice, 31 developed typical generalized leukemia (44%) after a latent period of 2½ to 16½ months (average age 9.6 months). In another series, filtered (through Sela filter candles) Ak leukemic extracts were used for inoculation of newborn C57BR/cd mice in 9 experiments, average age 7 hours[†] at time of inoculation. Of 42 inoculated mice, 14 developed typical generalized leukemia (33%) after a latent period of 10 to 14 months (average age 12.3 months). These mice developed pea-size peripheral lymph nodes, particularly prominent around the neck and in the axillary pits; they had enormous spleens and livers, large cylindrical, white and friable mesenteric, and frequently also mediastinal, lymphoid tumors. There was no evidence of any new growth at site of initial subcutaneous inoculation. The bone marrow, on smears, showed infiltration with lymphoblasts. Microscopic sections of livers showed infiltration with leukemic cells, particularly prominent around the larger vessels. The white cell blood count varied, in most instances, from 17250 to 209600/cu mm. Smears showed abnormal, immature white cells (1% to 10%) and predominance of lymphocytes (55% to 83%). Occasionally (2) the peripheral white blood count appeared normal, even though the sacrificed mice had a typical leukemia (large spleens and livers, and enlarged peripheral lymph nodes). As controls, in both experimental series, 43 newborn litter-mates were inoculated simultaneously with heated (67° to 80°C for ½ to one hour) Ak leukemic extracts; 3 litter-mates were untreated. Of 30 control litter-mates, injected with centrifuged heated extracts, 9 died at 9½ to 15 months of age without signs of leukemia; the remaining 21 are in good health, 15½ to 19 months old (average 18 months). Of the 16 control litter-mates injected with filtered heated ex-

tracts, or non-treated, 7 died without signs of leukemia when 9½ to 15 months old; the remaining 9 are well, at 11 to 17 months of age (average 14 months).

Further transplantation of leukemia developing in C57BR/cd mice after inoculation of either centrifuged or filtered Ak leukemic extracts. Leukemic cell suspensions prepared from livers, spleens and mesenteric tumors of those C57BR/cd mice that had developed leukemia as a result of inoculation of either centrifuged or filtered Ak leukemic extracts, were inoculated intraperitoneally (0.25 to 0.5 cc) into adult, i.e., more than 2 months old, mice of the C57BR/cd, Ak, C58, and C3H inbred lines. In each of 15 individual experiments, a different leukemic C57BR/cd donor mouse was used for the preparation of leukemic cell suspensions. Thirty-seven C57BR/cd mice were inoculated, and 30 developed leukemia after average incubation time of 31 days. Only 4 out of 35 Ak females developed leukemia 77 days after inoculation. None of the inoculated 12 C58, or 19 C3H mice developed leukemia. Thus, the C57BR/cd leukemic cell suspensions could, in most instances, be readily transplanted to adult mice of the C57BR/cd inbred line, but not to other strains; they could not be transplanted back to adult mice of the donor Ak strain. Only in a single transplantation experiment did 4 Ak females develop leukemia following implantation of the C57BR/cd leukemic cells. In these 4 mice, however, leukemia developed after a latency of 77 days; these females were then almost 6 months old, when leukemia may occur spontaneously. It is therefore questionable whether these 4 Ak mice developed leukemia spontaneously, or as a result of implantation of the C57BR/cd leukemic cells.

Discussion. Suckling, less than 2-day-old mice of the C57BR/cd inbred line were highly susceptible to inoculation of Ak leukemic cells; mice 4 to 14 days old were, in some instances, still susceptible, but the 20-day-old mice were already resistant. Adult (2 to 11 months old) C57BR/cd mice were completely resistant to inoculation of large (0.5 to 5 cc) doses of Ak leukemic cells. Following inocu-

[†] Only newborn C57BR/cd mice a few hours old were used for inoculation of centrifuged or filtered leukemic extracts, because previous experiments (3-5) suggested that susceptibility of newborn mice to inoculation of cell-free Ak leukemic extracts diminishes rapidly after the first 12 hours of life.

lation of Ak leukemic cells into newborn C57BR/cd mice, a leukemic tumor (lymphosarcoma) usually appeared at site of inoculation, followed within 2 to 4 weeks, by a generalized leukemia. Such transplanted leukemia consisted of Ak cells. The new foreign host served as a live medium, sufficiently susceptible to provide favorable conditions for multiplication of implanted Ak leukemic cells. This was evident from further experiments which showed that such transplanted leukemic tumors could be readily grafted back into adult mice of the Ak donor strain, but not usually into those of the C57BR/cd line.

The results were different when cell-free Ak leukemic extracts were used for inoculation. Thus, when either centrifuged or filtered Ak leukemic extracts were inoculated into newborn C57BR/cd mice (average age 6 to 7 hours[†]) generalized leukemia developed after a considerable delay, varying from 2½ to 16½ months, with no evidence of new growth at site of initial subcutaneous inoculation. Leukemic cell suspensions prepared from livers and spleens of such animals, produced acute leukemia when transplanted into adult mice of the same (C57BR/cd) strain, but could not be grafted, with rare exceptions, back to adult mice of the Ak inbred line.

These findings suggest that following inoculation of centrifuged or filtered Ak leukemic extracts into newborn C57BR/cd mice, the cell-free agent, introduced into the recipient strain, acted on susceptible cells of its new host in such a manner that after several months, leukemia resulted. This induced leukemia consisted, however, of its new host's own cells, *i.e.*, it was a C57BR/cd leukemia.

It has not yet been determined whether mice of the C57BR/cd inbred line, once inoculated with the Ak leukemic extracts, will be sufficiently susceptible to pass the acquired leukemic agent to their own untreated offspring, and then from one generation to another. Twenty-two males and females of this line, that had been inoculated with Ak leukemic extracts within a few hours after birth, were mated after they reached sexual maturity, and when in good health. Among their 147 untreated offspring, only 10 have thus far developed leukemia at 7 to 17 months

of age. The remaining 137 mice are now only 12 to 17 months old. Additional time is needed to complete this series of observations. These preliminary results, though incomplete, indicate that mice of the C57BR/cd inbred line, because of limited susceptibility, may occasionally transmit to their offspring an experimentally acquired Ak leukemic agent.

When filtered (Berkefeld, N, or Selas 03) (6,7), or centrifuged and then diluted(8), Ak leukemic extracts were inoculated into newborn C3H mice, some animals developed leukemia, and others, parotid gland carcinomas. When, however, filtered or centrifuged, Ak leukemic extracts were inoculated into C57BR/cd mice, typical generalized leukemia appeared in some inoculated animals, but none developed parotid gland carcinomas. Although the true cause of development of salivary gland carcinomas among C3H mice, inoculated with Ak leukemic extracts, has not yet been fully clarified, it now appears that these tumors can be induced only in certain strains of mice, such as the C3H inbred line. Thus, leukemic extracts which readily induced salivary gland carcinomas in C3H mice, did not cause development of such tumors when injected under apparently identical experimental conditions into mice of the C57BR/cd inbred line.

The results reported in this study are of interest because they confirm the presence of a filterable and thermolabile, cell-free agent in the Ak leukemic extracts. Whereas it was shown that this agent is pathogenic for C3H mice(1-8), and also for mice of the C57 black inbred line,[‡] causing, when inoculated under

[‡] In a preliminary series, 44 C57 black mice were inoculated when less than 12 hours old with Ak leukemic extracts centrifuged at either 3000 rpm (1400 x g) or at 9500 rpm (7000 x g), and 17 of them (39%) developed typical generalized leukemia at ages varying from 11½ to 22 months (average 17 months). Since less than 10% of untreated very old mice of the C57 black inbred line developed spontaneously lymphatic tumors(10), these data suggest that the C57 black mice developed leukemia as a result of inoculation with the Ak leukemic agent. Because of the relatively high incidence of spontaneous lymphatic tumors in mice of the C57 black line, however, we discontinued using these mice for inoculations with the Ak leukemic ex-

certain conditions, the development of leukemia, this study shows that the same agent is also pathogenic for newborn mice of the strain C57BR/cd. The Ak leukemic agent is therefore apparently pathogenic for mice of certain susceptible foreign strains, such as the C3H, C57BR/cd, and C57 black, provided that it is inoculated into the new hosts within a few hours after birth.

Since similar observations have been made with filtered extracts prepared from C58 leukemic donors(8,11), it now appears that both the Ak and the C58 spontaneous mouse leukemias are caused by filterable, thermolabile agents which, under certain conditions, can also be transmitted, by inoculation, to other strains of mice. In nature, however, these agents apparently would be transmitted in certain families of mice from one generation to another directly through the embryos(3,4,11). From an etiological and epidemiological point of view, mouse leukemia would be essentially similar to chicken lymphomatosis, also caused by a filterable agent(12) transmitted from one generation to another directly through the embryos(13). Since these causative agents are presumably of viral nature, both mouse leukemia(14), and chicken lymphomatosis (15) could be considered as "egg-borne" virus diseases.

Summary. 1. Cell suspensions prepared from Ak leukemic donors were inoculated into newborn C57BR/cd mice. Of 33 mice inoculated when less than 2 days old, 29 developed leukemia within 2 to 4 weeks. Of 61 inoculated when less than 14 days old, 14 developed leukemia, but of 17 injected at 20 to 24 days of age, none developed leukemia. Fifty-three adult C57BR/cd mice over 2 months old were inoculated with large doses of Ak leukemic cell suspensions, and all were resistant. 2. Leukemia developing in suckling C57BR/cd mice as a result of implantation of Ak leukemic cells, could be readily grafted to adult mice of the donor Ak strain, but not into recipient C57BR/cd line. 3. Centrifuged (7000 x g) Ak leukemic extracts were inoculated

tracts. Instead, mice of the C57 brown strain, subline cd, (C57BR/cd), essentially free from spontaneous leukemia, have been selected for this purpose.

into 71 newborn C57BR/cd mice (average age 6 hours), and 44% of them developed leukemia at average age of 9.6 months. Filtered (Selas) Ak leukemic extracts were inoculated into 42 C57BR/cd mice at average age of 7 hours, and 33% of them developed leukemia at average age of 12.3 months. 4. Forty-three litter-mates serving as controls, were inoculated simultaneously with heated (67° to 80°C for ½ to one hour) Ak leukemic extracts, and 3 were untreated, but none developed leukemia. 5. Cell suspensions prepared from those C57BR/cd mice that developed leukemia as a result of inoculation of either centrifuged or filtered Ak leukemic extracts, reproduced, in most instances, within a few weeks, acute leukemia, when transplanted into adult mice of the C57BR/cd (recipient) line, but could not be grafted, with rare exceptions, to adult mice of the Ak (donor) strain. 6. When, in previous experiments, filtered Ak leukemic extracts were inoculated into newborn C3H mice, some of the inoculated animals developed leukemia, and others developed parotid gland carcinomas. When, however, filtered Ak leukemic extracts were inoculated into C57BR/cd mice, typical leukemia developed in some of them, but none developed salivary gland carcinoma. 7. Twenty-two C57BR/cd males and females, that had been inoculated with Ak leukemic extracts within a few hours after birth, were mated after they reached sexual maturity, and in good health. Among their 147 untreated offspring, 10 have thus far developed leukemia at 7 to 17 months, suggesting transmission of leukemic agent from inoculated parents to untreated offspring.

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Phosphorylation in Cardiac Muscle from Failing and Unfailing Heart-Lung Preparations.*† (21218)

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(Introduced by M. H. Seevers.)

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Little is known about the sequence of biochemical events that leads to failure of heart muscle either in the intact animal or in isolated preparations. In an attempt to elucidate the cause of failure, most of the studies have been centered around the various enzymatic steps in the breakdown of carbohydrate and the concomitant generation of energy, primarily because more is known of these biochemical pathways. One approach to the problem of whether or not failure is associated with a defect in energy production has been the analysis of the high-energy phosphate stores of the normal and failing heart(1,2). The results of these studies have indicated that in failure, cardiac muscle retains its normal level of phosphocreatine and adenosine triphosphate. However, the level of the high-energy phosphate compounds of the heart is in reality merely the balance between the generation and the utilization of these compounds. Thus, an inhibition of both generation and utilization might still result in normal levels of high-energy compounds. It was therefore decided to study in a more direct way the ability of failed and unfailed cardiac muscle to generate energy-rich bonds.

Methods. In the control group of animals, mongrel dogs were anesthetized and immedi-

ately sacrificed, or the failed heart from the dog heart-lung preparation was employed. The animals sacrificed immediately were anesthetized with ether or pentobarbital, while preliminary operations on the heart-lung preparation were performed under pentobarbital anesthesia. The failed hearts in all experiments were obtained from the dog heart-lung, prepared by a modification of the Krayer-Mendez method(3). Failure in these animals was either spontaneous or pentobarbital induced. The competence index as described by Wollenberger(1) was used to evaluate the degree of failure. Those preparations having indices of from 0.8-1.0 were considered to be unfailed, while those with indices of from 0.0-0.2 were taken to be failed. In both failing and unfailing animals the left ventricle was rapidly excised and placed in 0.25 M sucrose solution. Mitochondria were then prepared by a modification of the method of Langemann *et al.*(4). Operations from this point on were carried out in ice baths in order to maintain a temperature of from 3-5°C. The tissue was then minced, rapidly weighed and a 10% homogenate prepared in isotonic sucrose using an all-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged for 5 minutes at 700 x g in a refrigerated centrifuge. The residue was discarded and the supernatant fluid was centrifuged at 12000 x g for 15 minutes. The resulting residue was resuspended in 0.25 M sucrose and recentrifuged at the same speed. The final

* Supported in part by a grant from the Michigan Heart Assn.

† Part of this paper was presented at the Am. Soc. for Pharmacol. and Exp. Therap., New Haven, Sept. 1953.

residue was made to volume with sucrose and added to the reaction mixture. The total volume in the Warburg flask was 1.8 ml. The final concentrations of the components of the reaction mixture, added in the order given, was as follows: 1.7×10^{-2} M Na-glycylglycine, pH 7.4, 2.2×10^{-2} M K-phosphate buffer, pH 7.4, 1.7×10^{-2} M KCl, 1.3×10^{-2} M $MgCl_2$, 2.8×10^{-3} M Na-adenosine triphosphate, 3.1×10^{-3} M glucose, 5.6×10^{-4} M disphosphopyridine nucleotide, 1.4×10^{-2} M Na-pyruvate, 2.2×10^{-3} M Na-malate, 9.2×10^{-6} M cytochrome c, 0.1 ml yeast hexokinase 3a(5), 60 units; 1.4×10^{-2} M Na-fluoride, 0.4 ml mitochondria in 0.25 M sucrose containing approximately 0.80 mg nitrogen. 8×10^{-3} M Na-succinate or 1.2×10^{-2} M α -ketoglutarate were also used as substrates. The sidearm contained 0.2 ml of 50% trichloroacetic acid to stop enzymatic activity at the end of the desired incubation period. Center well contained 0.2 ml of 2 N K-hydroxide, the gas phase was air and incubation temperature 25°C. Equilibration time was 5 minutes and incubation time varied from 10-30 minutes depending upon activity. Most experiments were run in triplicate. Oxygen uptake was measured by standard manometric methods and concomitant inorganic phosphate uptake as described (6). Phosphate to oxygen ratios (P:O) were then calculated from these values. Lindberg and Ernster (7) have substantiated the fact that the mitochondrial preparation when used with the hexokinase trapping system is a valid method of determining adenosine triphosphate synthesis.

Results. Table I summarizes results obtained when unailing dog cardiac muscle was studied. The P:O ratios in this group ranged from 2.04 (Exp. 4) to 3.07 (Exp. 7), average of 8 experiments 2.28. There seemed to be no difference in generation of high-energy bonds in tissues where the competence index was determined (Exp. 1-4) as compared with the values obtained from the myocardium of animals anesthetized and immediately sacrificed (Exp. 4-8). Nor did the nature of the anesthetic agent seem to alter the P:O ratio. Table II shows the results obtained when the dog heart-lung preparation was failing, mitochondria prepared from excised ventricle, and

TABLE I. Oxidative Phosphorylation in Cardiac Muscle from Unfailed Heart.* Eight experiments.

Anesthetic	Comp. index	Uptake		P:O \S
		Oxygen \dagger	Inorg. phos. \ddagger	
Pentobarbital	1.0	8.67	18.4	2.14
		9.37	20.0	2.14
		9.00	20.0	2.22
	.9	5.30	12.0	2.27
		4.64	9.6	2.08
	1.0	5.90	12.8	2.17
		5.54	12.0	2.16
		6.35	12.8	2.00
	.9	5.66	11.2	1.98
		5.75	12.0	2.09
		5.86	12.0	2.05
	—	6.27	14.4	2.30
		6.73	12.8	1.90
Ether	—	7.97	16.8	2.11
		8.17	16.8	2.09
		8.00	16.8	2.10
	—	4.02	12.0	2.98
		3.53	11.2	3.17
	—	5.54	14.4	2.60
		6.64	16.0	2.40

* Pyruvate plus malate substrate.

\dagger Microatoms.

\ddagger Micromoles.

\S Ratio of uptake of micromoles of inorganic phosphate to uptake of microatoms oxygen.

ability of tissue to synthesize high-energy bonds assayed. Here the P:O values range from 2.16 (Exp. 9) to 2.83 (Exp. 11), average 2.42. No difference was found between drug and non-drug failing animals. In comparing Table I and Table II there appears to be no difference in ability to generate energy as measured by means of the P:O ratio. Use of other substrates gave results similar to those obtained with pyruvate and malate, *i.e.*, no significant difference between failing and unailing muscle.

Discussion. Wollenberger (1) found similar phosphocreatine and adenosine triphosphate concentrations in hearts from normal and from failing heart-lung preparations. Greiner (2) analyzed papillary muscles that were moderately hypodynamic and found no fall in energy-rich compounds. The adenosine triphosphate level was decreased only in muscles which showed more than a 50% decline in systolic force. Furchgott *et al.* (8) indicated that normal guinea pig auricles have the same high-energy phosphate compound concentra-

TABLE II. Oxidative Phosphorylation in Cardiac Muscle from Failed Heart. Eight experiments.

Failure	Comp. index	Uptake		P:O
		Oxygen	Inorg. phos.	
Pentobarbital	.0	8.00	18.4	2.30
		5.98	12.0	2.01
		6.97	15.2	2.17
	.0	6.14	14.4	2.34
	.0	5.91	16.0	2.71
		5.54	14.4	2.60
		4.29	13.6	3.17
	.1	5.43	12.0	2.21
		5.42	12.8	2.36
		5.27	12.0	2.28
	.0	6.42	16.0	2.49
		5.68	17.6	3.10
		6.25	16.8	2.69
Non-drugs*	.0	8.36	19.2	2.41
		8.19	18.4	2.24
		7.83	19.2	2.45
	.0	9.76	23.2	2.38
		11.70	23.2	1.99
		11.20	24.0	2.14
	.0	6.62	16.0	2.41
		5.50	13.6	2.47

* Failed by occluding aorta.

† Spontaneous failure.

tions as either failing auricles or failing auricles reversed with strophanthin. All of the above data indicate that levels of high-energy compounds are not significantly different when muscles of failing and unfailing preparations are compared. The data, in no way, rule out the possibility that both production and utilization might be impaired in the hypodynamic muscles. Our work would suggest that the defect in the failing preparation is not one of energy production. It would also seem to indicate that future investigation into the mechanism of action of the cardiac glycosides at

sites of energy production will not prove fruitful.

In the interpretation of our data one should bear in mind that we are measuring oxidative phosphorylation in the presence of a fortified system. Any deficiency in quantity of substrate or other essential metabolite in a failing heart might well interfere with energy production. The evidence presented indicates that under optimum conditions the phosphorylative reactions in both failing and unfailing muscle are operating efficiently.

Summary. This investigation has demonstrated that there is no difference in phosphorylating ability, *i.e.*, enzyme concentration or activity, between normal cardiac muscle and similar tissue obtained from dog heart-lung preparations. These findings can be considered support for the thesis that if energy metabolism is concerned in failure, the defect is probably not one of production of energy-rich compounds.

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Nutritive Inadequacy of Whole Blood to Support Protein Repletion. (21219)

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The nutritive inadequacy of human hemoglobin, due to a primary deficiency of isoleucine, has been convincingly shown in studies with rats(1-3). Human plasma is also primarily deficient in isoleucine(4). Only fibrin, among the blood proteins, proved of highest value(5). As previously shown(6,7) protein-depleted rats avoid amino acid solutions which are devoid of one or more of the essential amino acids. Similarly an acid hydrolysate of whole hog blood was accepted only after fortification with the missing amino acids(8). The objectives of this study were to determine whether protein-depleted rats would accept whole human blood unfortified and fortified with the limiting amino acids (isoleucine and methionine). A fibrin hydrolysate of known nutritive value(9,10) was used as a standard.

Experimental. Whole blood. Whole human blood used was regular bank blood collected sterilely under vacuum in standard anticoagulant solution (Formula B, U.S.P. and N.I.H.). According to this formula each 100 cc of whole blood is made to contain dextrose 1.47 g, sodium citrate 1.32 g, and citric acid 0.48 g. Phenylmercuric nitrate, 0.002%, was added as preservative after bottle seals were broken. Blood was stored in refrigerator thereafter and removed only for use in feeding. By Kjeldahl analysis blood was found to contain 2.57% nitrogen. For feeding purposes 4.7 cc blood was diluted with water to 18-19 cc to provide 120 mg N/rat day. This dilution was chosen to produce a nitrogen content comparable to that of 5% fibrin hydrolysate. *Repletion assay.* Young adult male rats, 150-200 g were prepared for assay by 12-day depletion on non-protein diet and by orientation to protein solution feeding by the usual method(8). Basal diet contained: sucrose 40, dextrin 30, lard 18, corn oil 3, agar 2.5, haliver oil 0.5, wheat germ oil 0.25, choline chloride 0.3, and Jones and Foster salt mixture 4.0 g, thiamin • HCl 0.6, riboflavin 1.2, pyridoxine • HCl

0.6, calcium pantothenate 5.0, nicotinic acid 3.7, mixed tocopherols 2.5, folic acid 0.1, inositol 5 mg, and vit. B₁₂ 3 µg/100 g of diet. Water was supplied by drinking fountain, except during assay or drink trial when the test solution, or fibrin hydrolysate, were offered in place of water.

Acceptance trial. In the first trial 10 male young adult rats were selected which had been used for one 12-day assay of an experimental fibrin hydrolysate. These rats consumed the complete allotment of 5% fibrin hydrolysate, 0.24 g N/day, throughout the assay period and all gained weight at a rate greater than 4 g/day. Following this assay they were re-depleted for 12 days on non-protein diet. Rats prepared in this way show an inordinate interest in their drinking fountains and immediately drink some of any new solution offered them. When the diluted whole human blood was offered to 5 of these depleted rats, fairly good initial acceptance was shown. An average of 15.4 cc (range 9-18.4 cc) was consumed the first day. This was 83% of the allotment of 18.4 cc, equivalent to 120 mg N/rat day. Thenceforth, however, very little interest was shown in the blood. Fresh aliquots of diluted blood were offered daily, but the rats showed little interest in the solution. An average of only 2.6 cc (0-5 cc)/day was consumed for the next 8 days and the rats continued to lose weight at nearly the same rate as they had on non-protein diet. As positive control, a 5% fibrin hydrolysate was offered to the remaining 5 rats at 120 mg N/rat day. This was consumed completely by all rats within a few hours, and all 5 rats gained weight rapidly. This simple experiment clearly indicated that whole blood is not acceptable to the protein depleted rat. It remained to determine whether taste aversion or amino acid inadequacy of whole blood accounted for this failure.

Response to fortified blood. The 5 depleted rats which did not accept the whole human

blood, as described above, were repleted on stock diet for 10 days and again redepleted on non-protein diet for 12 days. They were then offered whole human blood to which had been added 1.5% *DL*-isoleucine and 0.32% *DL*-methionine. The latter were dissolved by cautious warming and agitation. By calculation these simple additions should correct the nutritive imbalance in the amino acid content of whole blood. Response of all 5 rats to this fortified whole blood was actually more prompt and dramatic than we had expected. Beginning with the initial offering all rats consumed the full daily allotment of 18.4 cc/day equal to 120 mg N. Intake of allotment was 100% for the 12 days feeding. Average weight gain was 15 g, range 7-20 g. These data indicate that nutritive inadequacy of whole blood is sufficient to account for its apparent distaste to protein-depleted rats.

A further test was made to compare fortified whole blood with 5% fibrin hydrolysate. Unfortified blood was included in this assay as a negative control. Fifteen male rats, 140-190 g, depleted for 12 days on non-protein diet, were prepared by the standard drink trial and divided into 3 equal groups. The 3 groups respectively were offered equal amounts of 5% fibrin hydrolysate, whole human blood, and whole blood plus 1.5% *DL*-isoleucine and 0.32% *DL*-methionine. All

solutions were adjusted with water so that 18 cc contained 120 mg N, the daily allotment.

Fig. 1 shows the weight changes for the 12-day feeding test. Acceptance of the whole blood alone was incomplete, as before. An average of only 5.6 cc/rat day was taken by this group. Again the rate of weight loss was only slightly less than when no protein was fed. Conversely, intake of allotment was 100% for the group which received the fortified whole blood and weight gain was almost as great as that for the fibrin hydrolysate.

Discussion. The severity of isoleucine deficiency was described by Rose *et al.* (11), who state, "no other type of amino acid deficiency produces in man so prompt and so profound a nutritive failure. On the 4th day of isoleucine deprivation the young volunteer subjects 'complained bitterly of a complete loss of appetite' and showed symptoms of nervousness, exhaustion, and dizziness which were exaggerated to a degree not observed before or since in other types of dietary deficiencies." In view of this one wonders about the effect of long continued injections of whole blood to patients unable to ingest isoleucine-containing foods in any form.

In spite of the lack of clear supporting evidence it is not uncommon to ascribe high nutritive value to both human plasma and whole blood. Traditional belief in the intrinsic nutritional value of whole blood and plasma received support from the extensive early work of Whipple *et al.* (12), which was carried out chiefly with dog blood, or blood components, in dogs. On the other hand, Robschey-Robbins *et al.* (13) reported that human globin failed to maintain weight and nitrogen balance in hypoproteinemic dogs, whereas dog globin did maintain weight and nitrogen balance. Miller (14) further demonstrated that only methionine supplementation to dog blood is needed for maintenance of N balance in dogs. By way of explaining these differences in nutritive value between human and dog hemoglobin, Brand and Grantham (15) then found that human hemoglobin contains 1.32% methionine, but no isoleucine. Conversely, dog hemoglobin contains by their analysis 0.42% methionine and 1.36% isoleucine. Both isoleucine and methionine would

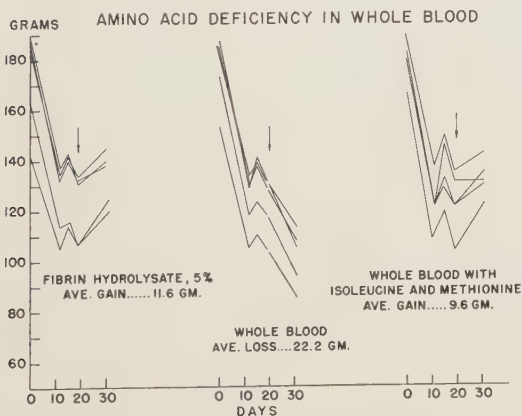


FIG. 1. Response of protein depleted rats to daily supplements of 3 test protein solutions. In each case the rats were depleted 12 days on non-protein diet and prepared for assay by 3-day drink trial on a standard hydrolysate followed by 3-day repletion. During assay rats were offered 18 cc equivalent to 120 mg N per day of respective solutions as shown.

obviously be needed to supplement human blood as the sole source of protein.

The value of plasma and whole blood for treatment of shock and for blood replacement is beyond question. On the other hand, they are poorly balanced nutritionally, and this fact should be clearly recognized. In a recent review Pollack and Halpern(16) describe some of the limitations of whole blood and plasma from the nutritive standpoint. These authors emphasize the fact that neither plasma nor blood are immediately available for nutritional purposes, but do not discuss the fundamental question of the adequacy of their amino acid make-up. Actually, however, we have been unable to find any report in the literature of the essential amino acid composition of whole human blood. The available data on individual amino acid content of certain of the constituent proteins of whole human blood are reviewed by Tristram(17). Amino acid analysis of whole human blood will be undertaken.

Summary. The inadequacy of whole human blood to serve as the sole dietary source of amino acids for repletion in the protein-depleted rat is demonstrated. Fortification of blood with isoleucine and methionine, the 2 indispensable amino acids in which blood is most deficient, largely corrects this nutritive inadequacy.

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Use of New Technic to Study Humoral Transmission of Hypertensive Effects of Vagal Stimulation. (21220)

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(Introduced by S. Ochoa.)

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We have previously shown(1-3) that stimulation of the central end of the cut cervical vagus nerve leads to the liberation of a hypertensive substance. The blood collected during the hypertensive phase has hypertensive effects under various experimental conditions. With 2 dogs linked in cross-circulation, vagus stimulation in one of them produces increased blood pressure in the other. This result is not modified by the insertion of a flow-regulating meter in the blood circuit between the 2 animals to avoid changes in pressure due to variations of flow. A number of observations suggested that the hypertensive substance, which resembles noradrenaline in pharmacological properties, is liberated from the arterial walls. Our results could not be explained by the assumption of Taylor *et al.*(4) that the hypertensive factor arises from the brain or the pituitary gland.

By use of a new cross-circulation technic we have confirmed our earlier results and obtained some further support for the arterial origin of the hypertensive substance.

Technic. The aortae of 2 dogs were sectioned and anastomosed with cannulae in such a way that the proximal end of the aorta of dog A was connected to the distal end of the aorta of dog B and conversely (Fig. 1). A flow regulator was inserted in the circuit and adjusted so that each dog lost as much blood as it received (90 to 110 ml/min.). The blood pressure was registered simultaneously in the femoral arteries or in both carotid and femoral arteries of each dog. The vagus was stimulated with an electronic stimulator as in previous experiments.

Results. *Effect of stimulation of central end of vagus.* Stimulation of the central end of the vagus of one of the dogs produces an increase in blood pressure in the carotid and femoral arteries of this dog followed by a smaller increase of pressure in the femoral artery of the second dog. There is no change

in the carotid blood pressure of the latter animal. The pressure increase is greater in the carotid than in the femoral artery of the stimulated dog. These results are illustrated in Fig. 2 and 3.

Effect of noradrenaline. Injection of noradrenaline (100 μ g) into the femoral vein of one of the dogs produces an increase of blood pressure in the carotid, but not in the femoral artery of this animal followed by an increase in the femoral arterial pressure of the second dog (Fig. 4). This result is easily understood as the injected noradrenaline passes through the central aorta to the femoral of the other dog before the blood reaches the injected dog's own femoral artery.

Discussion. The above results can best be interpreted by the assumption that stimulation of the central end of the vagus causes the liberation of a hypertensive substance (prob-

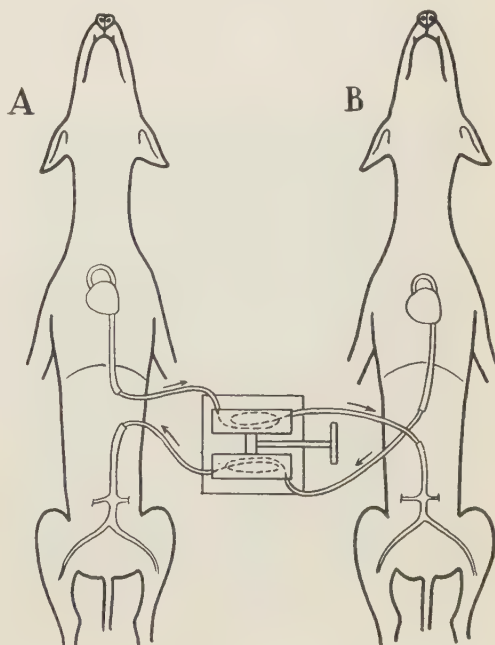


FIG. 1.
Diagram illustrating cross-circulation technic.

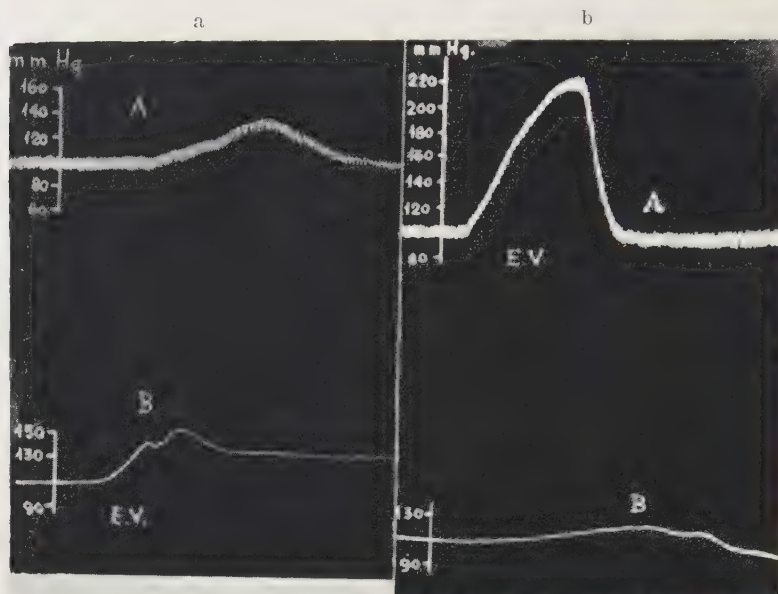


FIG. 2a. Blood pressure tracings showing effect of stimulation of central end of vagus of one dog. B. Femoral artery of stimulated dog. A. Femoral artery of the other animal. Fig. 2b. As 2a. A. Carotid artery of stimulated dog. B. Femoral artery of the other animal.

ably noradrenaline) from the arterial walls themselves. If stimulation led to the liberation of a substance of encephalic or hypophyseal origin (or derived from another organ) into the venous circulation one would expect the results of the stimulation to mimic

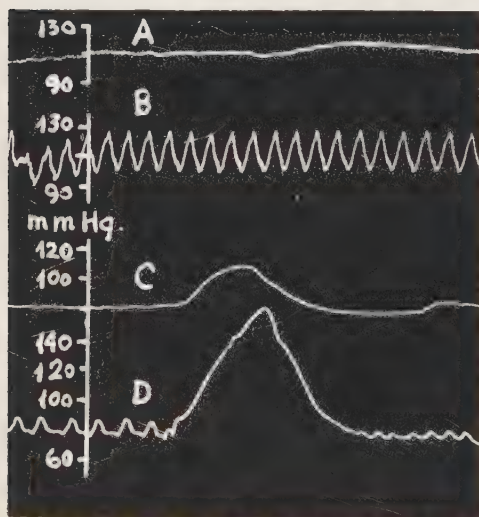


FIG. 3. As Fig. 2. C and D, femoral and carotid arteries respectively of stimulated dog. A and B, femoral and carotid respectively of the other animal.

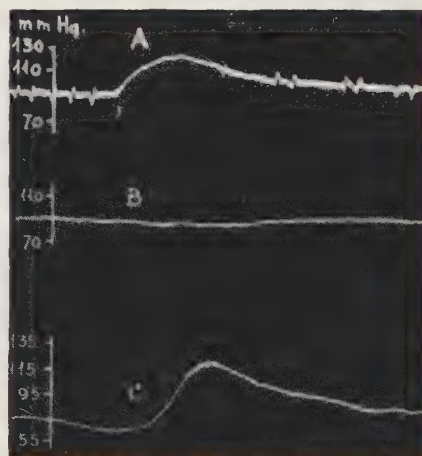


FIG. 4. Blood pressure tracings showing effect of intravenous injection of 100 μ g of noradrenaline into one dog. A and B, carotid and femoral artery respectively of injected dog. C, femoral artery of the other animal.

those obtained after the intravenous injection of noradrenaline, *i.e.*, increase of pressure in the carotid, but not in the femoral artery of the treated dog, followed by increase in the femoral pressure of the untreated one. One would not expect an increase in both carotid and femoral arterial pressures of the stimulated dog as was actually observed.

Summary. Experiments on dogs linked in cross-circulation, by connecting the central end of the aorta of one dog to the peripheral end of the aorta of the other and vice versa, have confirmed earlier results in this laboratory indicating that a hypertensive substance is liberated on stimulation of the central end of the cut vagus nerve. Comparison of the results of the vagus stimulation and noradrenaline injection have provided further evidence for the assumption that the hypertensive substance is probably liberated from the ar-

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Effects of Ouabain on Beat and Oxygen Consumption of Embryonic Chick Hearts.* (21221)

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Friedman and Bine(1) demonstrated that the embryonic duck heart is very sensitive to digitalis preparations. Preliminary experiments showed us that embryonic chick hearts, although not as sensitive to digitalis as embryonic duck hearts, are, nevertheless, satisfactory in this respect, easier to obtain, and less expensive. The cartesian diver technic is admirable for measuring metabolism of minute tissues. Further preliminary experiments showed that it could be adapted to measuring oxygen consumption of relatively large embryonic chick hearts weighing as much as 400 μ g, dry weight, after 6 days of incubation. In another communication(2) we describe the necessary basic modifications for measuring oxygen consumption of embryonic chick heart with the cartesian diver.

Methods. The cartesian diver with a shaker and divers with caps and mixers(2) was employed. Fertile chicken eggs were incubated 5 days, producing embryos with hearts of maximum size (80 to 300 μ g, dry weight), which would beat regularly and reliably. These hearts were dissected into Ringer or

other suitable solution until needed. The solutions were kept at 37°C. A heart was placed in the bulb of a diver, 10 mm³ of Ringer or other suitable solution was added. Air was replaced by 100% oxygen. Exactly one mm³ of same solution (control) or of a solution containing ouabain was deposited on surface of the neck, and a magnetic mixer placed in this droplet. After reoxygenating, adding the KOH seal, and placing a buoyed cap on the diver, it was immersed in the flotation medium in a diver holder. About 10 minutes was allowed for temperature equilibration at 37.3°C. Observations on rate and regularity of the beat were made on the odd numbered minutes, and measurements of suspension pressures made on even numbered minutes for 42 minutes, then at less frequent and less regular intervals up to 4 hours. Mixing was performed at 11 minutes as described elsewhere(2). Changes in suspension pressures were corrected for changes in barometric pressure and were multiplied by the diver constant to give oxygen consumption. At the close of each experiment the hearts were weighed on a quartz fiber balance; all oxygen consumption figures were converted to mm³/mg of dry weight. The results of comparable experiments were averaged.

* The authors thank the Eli Lilly Co., Indianapolis, Ind., for generous quantities of ouabain and for a grant which made this study possible.

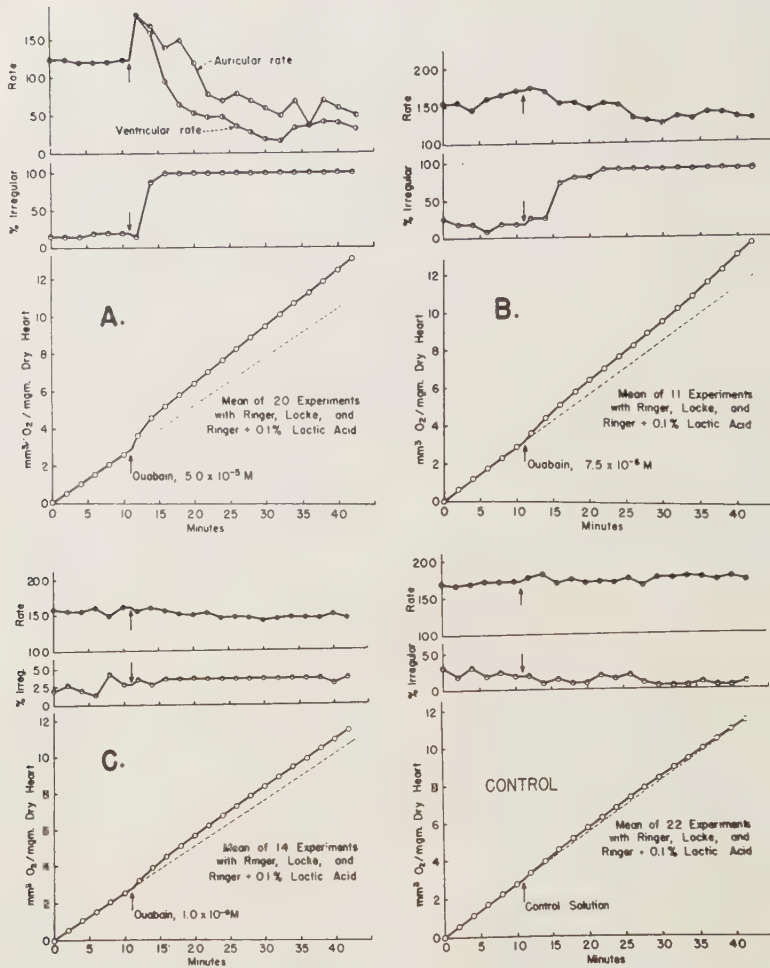


FIG. 1. Effects of ouabain on beat and oxygen consumption of embryonic chick hearts.

Results. A total of 108 experiments were performed. The following concentrations of ouabain were employed in Ringer, Locke, and Ringer plus 0.1% lactic acid at pH 7.2: 5.0×10^{-5} M, 2.5×10^{-5} M, 1×10^{-5} M, 7.5×10^{-6} M, 5×10^{-6} M, 2.5×10^{-6} M, and 1×10^{-6} M.

The results obtained with Ringer, with Locke, and with Ringer plus lactic acid were identical, and therefore, averaged; the results are shown in Fig. 1. For the sake of brevity only 3 concentrations are graphed; intermediate concentrations gave intermediate results.

Fig. 1A shows that when ouabain, at a concentration of 5.0×10^{-5} M, was added to the

hearts, there was an immediate increase in oxygen consumption that persisted throughout the period of observation. In addition, ouabain caused an immediate increase in heart rate, followed, after about 3 minutes, by the appearance of irregularities, and, simultaneously, a decrease in both auricular and ventricular rates, particularly the latter.

Figs. 1B and 1C show that when lower concentrations of ouabain were used, the increase in oxygen consumption persisted, although of smaller magnitude, but changes in the rate and production of irregularities diminished to zero.

Fig. 1 Control shows that oxygen consumption, heart rate, and incidence of irregularities

was not altered when control solution was mixed with control solution.

In some experiments the hearts were dissected directly into Locke solution containing one mg % prostigmine and 0.5 mg % acetylcholine, or Locke solution containing 2 mg % atropine. These drugs did not alter the heart rates nor the incidence of irregularities. After 30 minutes in these solutions, observations on rates, regularity, and oxygen consumption were begun. Ouabain, 5×10^{-5} M, was added at 11 minutes. In such experiments the increase in oxygen consumption produced by ouabain was not altered, but the effect on rate and incidence of irregularities produced by ouabain was exaggerated by prostigmine and acetylcholine and were reduced by atropine.

In many experiments, observations were continued over periods up to 4 hours. In all, including both control and ouabain experiments, there was a gradual decrease in oxygen consumption; the decrease could not be correlated with any previous treatment.

In several experiments, the hearts were kept at 37°C for one or 2 hours before being placed in divers. Deterioration, as indicated by a diminishing oxygen consumption, occurred, but the response to ouabain was not altered.

Discussion. That ouabain causes an increased oxygen consumption agrees with many previous observations(3). That ouabain causes irregularities is in accord with our predecessors.

Investigators(4,5) have reported an increase in oxygen consumption after digitalis in the preparations, such as heart slices, in which there is no beat nor visible muscular contraction. In such preparations the change in oxygen consumption can not be due to alterations in heart rate nor heart beat. Our results show that the time of onset of irregularities in a heart preparation that is capable of supporting a beat differs from the time of onset of increased oxygen consumption; furthermore, the minimum concentration of ouabain that produces irregularities differs from the minimum that causes increased oxygen consump-

tion. In addition, in a few experiments with prostigmine plus acetylcholine and with atropine, the severity of the irregularities may be modified without essentially altering the increase in oxygen consumption.

These results may be interpreted to mean that the increase in oxygen consumption after ouabain is not directly related to changes produced in the beat of the heart.

Summary and conclusions. 1. Ouabain, in concentrations from 1×10^{-6} M to 5×10^{-5} M, was added to embryonic chick hearts whose oxygen consumption was being measured in the cartesian diver. Controls, in which Ringer, Locke, etc., was mixed with Ringer, Locke, etc., respectively, were also employed. 2. Ouabain, in concentrations from 1×10^{-6} M to 5×10^{-5} M, inclusive, caused a prompt increase in oxygen consumption, roughly proportional to concentration. 3. Ouabain, at a concentration of 5×10^{-5} M, caused an immediate increase in heart rate, and, after about 3 minutes, irregularities in the beat of the heart. 4. Ouabain, in a concentration of 1×10^{-6} M, did not produce irregularities and did not alter the heart rate. 5. Oxygen consumption, heart rate, and incidence of irregularities were not altered by mixing control solutions with control solutions. 6. Acetylcholine plus prostigmine increased, whereas atropine decreased the severity of the irregularities produced by ouabain, without appreciably altering the increase in oxygen consumption caused by ouabain. 7. The increase in oxygen consumption after ouabain is not directly related to changes in the beat of the heart.

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Effects of 3-(o-Toloxo)-Propane-1,2-Diol (Mephenesin) on Sodium, Potassium, and Water Content of Frog Skeletal Muscle. (21222)

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The relaxing property of 3-(o-toloxo)-propane-1,2-diol, or Mephenesin, has led in the last 10 years to a revival of interest in this compound. Although reports of Mephenesin effects on muscle and peripheral nerve have been conflicting(1,2), it has been definitely shown that this drug decreases α -excitability of frog muscle(3). The well-established dependence of electrical properties on ion content and exchange suggested a study of sodium and potassium in muscle exposed to Mephenesin. For this study 2 procedures were followed: one consisting of analysis of muscle for sodium and potassium after a period of immersion in drug solution; the other involving the influence of Mephenesin on muscle weight changes resulting from immersion in modified Ringers solution.

Method. Semitendinosus and peroneous muscle pairs were removed from *Rana pipiens* and kept in Ringers solution at 5°C for 4 to 8 hours before the beginning of an experiment. This was found to minimize erratic weight changes often seen in muscles just after their removal from the animal. Ringers solution (R) was of pH 7.3-7.4; its exact composition has been described previously(3). NaCl was reduced to 55.5 mM per liter in hypotonic, sodium-poor Ringers ($\frac{1}{2}$ R) and in potassium-rich Ringers (KR), the KCl content being raised to 56.95 mM per liter in the latter case. All solutions then, except $\frac{1}{2}$ R and $\frac{1}{2}$ RD (hypotonic Ringers containing drug) were isotonic. Oxygen was bubbled for 5-10 minutes through all solutions into which the muscles were to be placed. Care was taken in all experiments to minimize trauma to muscles.

1. Weighing experiments. Each muscle was blotted on filter paper, weighed on a torsion balance, and placed in a small glass dish containing one of the test solutions. Four solu-

tions were used for immersion in each experiment: R, $\frac{1}{2}$ R or KR, RD, and $\frac{1}{2}$ RD or KR.D. Valid comparisons could be made only between paired muscles, and the experiments were arranged accordingly. Each muscle was weighed at intervals over a period of several hours, at the end of which time it was placed again in R. **2. Sodium and potassium analyses.** After initial weighing, one muscle of a pair was kept in Ringers, while the other was exposed to one of the test solutions. Muscles were allowed to remain at room temperature (18-20°C) in these solutions for various lengths of time, after which they were reweighed and placed in platinum crucibles for ashing. A flame photometer was used to analyze solutions of muscle ash for sodium or potassium. Experimental values were converted to milliequivalents of potassium or sodium per kg initial muscle wet weight, and comparisons were made.

Results. **1. Weighing experiments.** Table I shows that Mephenesin, even in relatively high concentration, had no effect on the weight increase resulting from immersion in KR. However, 0.3% Mephenesin caused a significant increase in the weight gain of muscle in $\frac{1}{2}$ R. This effect was reversible.

2. Sodium and potassium. The changes in potassium and sodium content of muscle brought about by immersion in $\frac{1}{2}$ R in the absence of drug are seen in Table II: there was a slight decrease in potassium, and a large decrease, amounting to about 30% of the normal muscle level, in sodium. It was found that the procedure of repeated handling and weighing of the muscles caused a significant gain of sodium, even in $\frac{1}{2}$ R. Further, in muscles so "handled" Mephenesin seemed to increase the sodium and decrease the potassium content.

Mephenesin, at a concentration of 0.1% in R, produced no significant changes in the sodium and potassium of undisturbed muscles.

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TABLE I. Effects of Mephenesin on Muscle Swelling.

Mephenesin conc. (%)	Exp. procedure	No. of exp.	Mean wt change (%)	Stand. error of mean	Probability
.05	KRD-KR	5	-1.06	± 2.35	.636
	RD-R	5	2.60	± 1.87	.181
.1	KRD-KR	7	-3.37	± 3.18	.289
	RD-R	7	-1.89	± 3.09	.531
	$\frac{1}{2}$ RD- $\frac{1}{2}$ R	4	-.96	$\pm .94$.300
	RD-R	4	-3.28	± 2.60	.221
.3	KRD-KR	2	-.05	$\pm .71$	1.000
	RD-R	2	.40	$\pm .57$.425
	$\frac{1}{2}$ RD- $\frac{1}{2}$ R	4	21.40	± 1.09	<.001
	RD-R	4	6.98	± 3.74	.097

Max % gain calculated from original wet wt for each muscle. Effects seen increased with time to max at 160 min. Means calculated from differences between paired muscles, one of which was immersed either in hypotonic, sodium-poor Ringers ($\frac{1}{2}$ R) or in potassium-rich Ringers (KR), the other in similar solution plus drug. Absence of drug effect in unaltered Ringers indicated by values of (RD-R). Avg % gain of muscle wt in R (22 exp.), 2.80, stand. error of mean, ± 5.89 ; in $\frac{1}{2}$ R (8 exp.), 27.22, stand. error of mean, ± 3.21 ; in KR (14 exp.), 20.21, stand. error of mean, ± 5.58 .

There was a small, questionably significant loss of potassium from drug-exposed muscles in $\frac{1}{2}$ R. This possibility of a potassium loss was investigated at a higher drug concentration: Table II shows a loss of approximately 20% of the normal cellular potassium produced by 0.3% Mephenesin, both in R and in $\frac{1}{2}$ R.

Discussion. The procedure of isolating and immersing frog muscles in Ringers solutions changes the water and electrolyte balance between fibers and extracellular space. The fact that *in vivo* chloride space values are considerably lower than those calculated from *in vitro* studies is a good indication of this(4,5). A certain amount of independence from such changes can be achieved by considering only differences between muscles of a pair; this was the practice in the present studies.

Muscle swells in a sodium-poor solution ($\frac{1}{2}$ R), losing a small amount of potassium and a much larger amount of sodium, around 30% of the normal content. If extracellular fluid is to be in equilibrium with immersion medium, and if 60% of the muscle sodium is found in the extracellular space, then a loss of 30% of the sodium is required from muscle in $\frac{1}{2}$ R. Any potassium loss might represent a tendency for muscle fibers to come into equilibrium with the lowered potassium concentration of the extracellular fluid. Muscle

also swells in potassium-rich Ringers (KR), even though isotonicity is maintained by an appropriate decrease of sodium. This indicates a high permeability to potassium. Any agent which could alter permeability to potassium should produce a change in this type of swelling. Shanes(6) found, for example, that cocaine markedly decreased the swelling of muscles in potassium-rich Ringers, and he concluded that potassium permeability had been decreased. In this respect Mephenesin had no effect: the swelling of muscles in KR was not altered. On the other hand, muscles in hypotonic, sodium-poor Ringers definitely swelled more in the presence of Mephenesin than in its absence.

This drug effect on hypotonic swelling is not easily explained. It cannot be interpreted as a decrease in potassium permeability, because Table II shows a greater K loss in Mephenesin ($\frac{1}{2}$ RD) than in $\frac{1}{2}$ R. One could speculate that Mephenesin diminished the effect of the sodium pump, thus allowing accumulation of the ion and accompanying water inside the cell. But were this the case, drug-exposed muscles should have swelled in isotonic Ringers solution as well, and this did not occur.

Since the study of swelling involved frequent weighings, the question was raised of changes which might result from such "han-

TABLE II. Effects of Mephenesin on Sodium and Potassium Content of Frog Muscle.

Mephenesin conc. (%)	Exp. procedure	No. of exp.	Mean change (meq/kg muscle)	Stand. error of mean	Probability
No drug	$\frac{1}{2}$ R -R	9	K: -7.0 Na: -17.9	± 3.3 ± 5.0	.080 .011
.1	RD-R (not handled)	5	K: 2.4 Na: -5.1	± 2.5 ± 2.1	.392 .104
	RD-R (handled)	6	K: -1.5 Na: 6.6	± 1.2 ± 1.3	.267 .003
	$\frac{1}{2}$ RD- $\frac{1}{2}$ R (not handled)	5	K: -3.2 Na: -0.3	± 1.6 ± 1.4	.119 .839
	$\frac{1}{2}$ RD- $\frac{1}{2}$ R (handled)	6	K: -4.8 Na: 3.1	± 1.8 ± 1.3	.046 .063
.3	RD-R	9	K: -14.1	± 3.9	.007
	$\frac{1}{2}$ RD- $\frac{1}{2}$ R	9	K: -16.6	± 3.6	.002

Mean change arrived at by averaging differences obtained in muscle pairs, one of which had been immersed in Ringers (R) or in hypotonic, sodium-poor Ringers ($\frac{1}{2}$ R), the other of which had been exposed to Mephenesin in R or $\frac{1}{2}$ R. Control value was subtracted from experimental (e.g., $\frac{1}{2}$ R-R), so that positive mean change indicates gain by experimental muscle, negative change, loss. Initial wet wt of muscle used in all calculations of meq change/kg muscle.

dling". Muscles disturbed or handled at intervals during the course of a 2- to 3-hour period were seen to gain sodium; muscle weight was also increased. Since in Ringers solution this procedure produced an increase in muscle sodium but no definite change in potassium, it is probable that only extracellular volume increased. Extracellular fluid, being in equilibrium with the immersion fluid, would gain sodium for the muscle by means of volume increase. It is of interest, however, that Mephenesin appeared to intensify this increase in intracellular fluid and sodium (probability: .003).

The weight gains produced by handling confirmed results of similar experiments by Fenn(7). By showing that values for chloride space were uniformly larger whenever muscle weights were larger, Fenn produced good evidence that such weight changes truly represented increases in extracellular fluid.

In Ringers solution no significant potassium change was seen at the end of 2.7 hours in the presence of 0.1% Mephenesin as compared to the control. A small, questionably significant loss of sodium was seen; this amounted to less than 10% of the usual muscle content. In $\frac{1}{2}$ R a similar lack of effect on sodium content was noted. As can be seen from Table II, the loss of sodium in $\frac{1}{2}$ R in the absence of drug amounted to 17.9 meq/kg or

roughly 30%; Mephenesin did not alter this loss.

Although there was no statistically significant change in potassium caused by 0.1% Mephenesin in $\frac{1}{2}$ R, the fact that each of 5 muscle pairs showed a small loss (mean: -3.2 meq/kg, probability: .119) suggested further studies at a higher drug concentration. There was a remarkable loss of potassium when muscles were immersed in 0.3% Mephenesin both in R and in $\frac{1}{2}$ R. Muscles in this concentration of drug in $\frac{1}{2}$ R took up water to the extent of almost 25% of initial muscle weight. This is indicated in Table I, in which the possibility of drug osmotic effect has been considered. The cause of such large potassium losses might be attributed to: 1. *Muscle stimulation by drug*. Mephenesin is known to initiate contracture(8), and perhaps this property, manifested over a period of several hours was capable of producing this large potassium loss. Although there was no visible twitching of these muscles, and no length or tension measurements were made, conceivably some shortening did occur. Muscle stimulation generally results in an exchange of potassium for sodium and a gain in intracellular and extracellular water(9). 2. *Release of bound or normally non-diffusible potassium*. Much has been made of the ability of myosin to bind potassium and of the dependence of this

process on pH(10). It is conceivable that any potassium which might be bound in muscle at rest or in the "normal" state becomes free and diffuses out as a result of drug action. The idea that such a release occurs when contraction takes place is far from new(11). 3. *Change in muscle membrane permeability.* In the absence of contraction, this possibility, that the cell is simply made more permeable to potassium, is unlikely. The fact that muscle swelling was not altered by the drug in KR lends improbability to such a notion.

It is possible that more than one mechanism was operative here. If as much as 33% of muscle potassium is bound or non-diffusible (12), then it is possible that some of the 20% loss came from this fraction. Since contraction can be produced by the drug, the changes related to normal function were undoubtedly reflected in the results above (the usually-seen potassium loss, sodium and water gains, and associated permeability changes).

Summary. Mephenesin in concentrations up to 0.3% caused no change in the swelling resulting from immersion in high-potassium Ringers. Mephenesin at a concentration of 0.3%, but not at 0.1%, caused a large, reversible increase in swelling resulting from immersion in sodium-poor, hypotonic Ringers. This relatively high drug concentration also caused a 20% loss of muscle potassium both in regular Ringers and in hypotonic, sodium-poor Ringers. These changes are thought to

be related to muscle contraction produced by Mephenesin. Confirming results of similar experiments by Fenn(7), the procedure of disturbing or handling muscles at intervals throughout an experiment was seen to result in muscle weight gain, a small sodium increase, and little alteration of potassium content; this process was intensified by Mephenesin.

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Effect of Air Ions on Succinoxidase Activity of the Rat Adrenal Gland.* (21223)

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The atmosphere contains at all times

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variable amounts of gaseous molecules that possess a negative or positive charge. The concentration of these "air ions" under normal conditions may vary between 400 and 2000 ion pairs/cc, but under certain conditions an excess of either positively or negatively charged ions may occur. The possible physiologic effect of air ions has interested a number of investigators. In a series of studies carried

out with human subjects exposed to ionized atmospheres containing an excess of positive or of negative ions(1,2), it was reported not only that physiologic effects were demonstrated but that differences were observed in the response to an excess of positive as compared to negative air ions. In the period intervening between these early experiments and the present, improved methods have been devised for the generation of air ions and their separation so as to produce an excess of ions of either positive or negative polarity. Skilling and Beckett(3) have recently described a method for the production and separation of positive and negative air ions. The ionization of the air is accomplished by alpha particles from radioactive polonium 210. Ion separation is then brought about by the use of a charged screen adjacent to the polonium emitter. In the electrostatic field thus produced, the ions oppositely charged to that of the screen are attracted toward the electrode and discharged. Those ions having the same charge as the screen are repelled and travel into the air to produce an excess of positive or negative ions, depending on the charge originally placed on the screen. This ionizing device has been used to produce controlled ion atmospheres in studies of the physiological effects of such atmospheres on experimental animals. Worden(4) maintained hamsters in ionized air at concentrations of approximately 1600/cc of one polarity and 200 of the opposite. He reported that the carbon dioxide capacity of the plasma was significantly increased in those animals kept in negatively ionized air as compared to those in positively ionized air or in a normal atmosphere. The blood pH was also elevated in the negatively ionized group(5). Blood samples for analysis were drawn from individual animals sacrificed at 24, 48, 72 or 96 hours after ionization had begun. The changes reported were observed at the end of the first (24-hour) period, as well as in each of the subsequent periods. Later work showed that the effects could be demonstrated after a one-hour exposure and, furthermore, that the plasma chloride concentrations varied inversely with the CO₂ capacity.

Several investigators have reported on evi-

dence for increased activity of the adrenal cortex in animals exposed to ionized air. Holloway(6) investigated the changes in cholesterol and ascorbic acid which occurred in the adrenals of rats maintained for varying periods of time up to 72 days in positively or negatively ionized air. Rinfret and Wexler(7) using histological methods for studies of adrenal changes found that exposure to an atmosphere containing an excess of positive ions (2500-3000/cc) produced histological changes indicative of elaboration by the glands of both salt and carbohydrate-regulating corticoids. In this paper we are reporting the results of an investigation of the succinoxidase activity of homogenates from the adrenal glands of rats maintained in ionized air. McShan *et al.*(8) in examining the succinoxidase activity of homogenized endocrine tissues found that the QO₂ of the adrenals was higher than that of any other endocrine tissue studied and they suggested that the succinoxidase content could be correlated with the activity of the gland.

Methods. Male Sprague-Dawley rats, 200-240 g in weight were used. For analyses of the succinoxidase activity of the adrenal glands, the animals were sacrificed by rapid decapitation. Both glands from a given animal were removed as quickly as possible, chilled, trimmed and homogenized for one minute in 2 ml of ice-cold triple distilled water using a Potter-Elvehjem all-glass homogenizer. The homogenate was diluted to 4 ml and stored in the freezing compartment of the refrigerator until used for assay (usually within less than one hour). The method of Schneider and Potter(9) was employed for the manometric assay of succinoxidase. Oxygen uptake served as a measure of the activity of succinoxidase in the homogenate. Nitrogen determinations were made on one-ml aliquots of the homogenate by a micro Kjeldahl method(10) and the oxygen uptake was then expressed as QO₂, N, *i.e.*, μ l oxygen uptake per mg tissue nitrogen per hour. Preliminary control experiments suggested that the succinoxidase activity of the adrenal was readily affected by a number of extraneous stimuli such as light, temperature, noise, increased activity within the animal room, frequent handling of

the animal, and the feeding and weighing schedule. Attempts were made to control these variables as closely as possible; the illumination in the animal room was confined to daylight from a light well; the temperature of the room was maintained at 80°F; an effort was made to select only those animals which seemed well adjusted to their surroundings and each test animal was isolated from the group for at least 4 days prior to a given experiment.

Results. In consideration of the diurnal variations in the activity of the animals, it was of interest to compare the succinoxidase content of the adrenal gland observed at periods of minimum and maximum activity. The following results were obtained in the course of experiments conducted during the summer season (July and August). The succinoxidase of the adrenal homogenates obtained from normal rats during a period of minimum activity (day) averaged 175 (± 8.5)[§]; during a period of maximum activity (night), 226 (± 19.3). The difference, which represents a 52% increase in activity, is statistically significant (P value less than 0.01). It is apparent from these data that the activity status of the animal exerts an important effect on the succinoxidase content of the adrenal gland of the rat. The change from minimum to maximum activity may occur in a very short period of time. Any sudden disturbance in the animal room or the repeated withdrawal of animals from a group in a single cage, for example, would alter the activity of the animals and cause a rise in succinoxidase to a maximum. In an attempt to minimize this experimental variable our studies were carried out during the period of maximum activity when slight disturbances within the animal room would be less likely to cause a further increase in the activity of the animal.

Ionization of the air in the animal cage was accomplished by the installation on the roof of a 7 x 15 x 10 inch metal cage of the tubular

TABLE I. Succinoxidase Activity of Homogenates of Adrenal Glands from Rats Subjected to Various Stimuli. Six experiments.

Group	Succinoxidase (QO_2 , N*)	Deviation from control (%)	Significance of deviation, P values (11)
Controls (8)†	375 (± 16.3)	—	—
Shock (4)	551 (± 35.9)	+46.9	<.01
Positive ions (9)	325 (± 39.9)	-13.3	<.01
Negative " (10)	389 (± 42.8)	+ 3.7	.40
ACTH (5) (5 mg 4 hr previously)	415 (± 36.7)	+10.6	.02
ACTH (6) (5 mg b.i.d. x 6)	307 (± 37.0)	-18.1	<.01

* Mean values for μl oxygen uptake/mg tissue nitrogen/hr, including stand. error of each mean.

† Numbers in parentheses refer to number of animals in each group.

type ionizer described by Skilling and Beckett(3). Ionization of molecules in the air was produced by the alpha emissions from a polonium foil. The type of ionization which predominated was controlled by the charge placed on the outer metal tube of the ionizer. This charge was altered in accordance with the connection made to the appropriate terminal of a 300-volt B battery. Ion density was measured by the use of an ion collector in combination with a Beckman Ultrameter. The concentration of small ions (*i.e.*, ions with the highest mobility) in the air of the negatively ionized cages averaged 4500-6000 negative ions/cc and 300-400 positive; in the positively ionized cages, 12000-17000 positive ions/cc and 200-250 negative. The average mobilities of the negatively charged ions are higher than the positive. As a result, the negative ions were absorbed on the walls of the cage more rapidly than the positive. This fact accounts for the lower ion density in the negatively ionized atmospheres.

The series of experiments reported in Table I was conducted during an 8-week period in November and December. It will be noted that the succinoxidase activity of the control animals is significantly higher than had been found in the experiments on the effects of activity on adrenal succinoxidase reported above. These were performed during the summer months. It is possible that this is a

[§] QO_2 , N; *i.e.*, μl oxygen uptake/mg tissue nitrogen/hr. Figure in parentheses is standard error of mean

$$\text{calculated as S.E.} = \sqrt{\frac{\sum d^2}{n}}$$

reflection of seasonal changes in adrenal succinoxidase activity, since no other alteration in the experimental conditions or environment occurred during these 2 periods.

In the first experiments with positively or negatively ionized atmospheres, the succinoxidase activities were found to be very high. It was also noted that soon after installing the ionizer in the cage, the animal in the course of investigating this new addition to his surroundings invariably received an electric shock from the ionizing equipment. The polonium foil was then removed from the ionizer and the experiment repeated. Although no ionization was now present, the enzymatic activity 4 hours after installation of the ionizer without a polonium element was still significantly elevated (Table I). The results of this experiment served not only to modify the method for installation of the ionizer on the cage but also indicated the extent to which the succinoxidase activity of the adrenal would reflect the imposition of a stress stimulus. Following these experiments the ionizers were raised above the roof of the cages with a plastic cylinder, 5 inches in height. This device prevented direct contact of the body of the animal with the ionizer.

Animals were maintained in an atmosphere of positively or negatively ionized air for 4 hours. The effects of the ionized atmospheres on the adrenal succinoxidase activity are shown in Table I. In the case of those animals maintained in the positively ionized air, a statistically significant decline in succinoxidase activity was found, an effect opposite to that of the stimulus of an electric shock. Negatively ionized air produced a very slight rise which under the conditions of these experiments was not statistically significant. For comparison, the effect on adrenal succinoxidase measured 4 hours after the single subcutaneous injection of 5 mg of ACTH

(Armour powder), or of 5 mg ACTH twice a day for 6 days was studied. The single ACTH injection produced a moderate rise in succinoxidase whereas after the repeated injections a significant fall was noted.

Summary. The effect of air ions on the succinoxidase activity of the rat adrenal gland has been studied. The activity status of the animal was found to affect the succinoxidase concentration of the adrenal. Electric shock produced a very significant rise. After 4 hours in positively ionized air the succinoxidase content of the rat adrenal was significantly reduced; a similar period in negatively ionized air produced a slight but insignificant rise. By comparison, a single injection of 5 mg of ACTH produced a moderate rise whereas the same dose given twice daily for 6 days resulted in a significant fall in adrenal succinoxidase.

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On the Problem of Naturally Occurring Aberrant Strains of *Brucella*. (21224)

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Recently, increased attention has been paid to the isolation of naturally occurring *Brucella* strains which do not meet the established criteria for differential diagnostic characteristics of *Brucella suis*, *B. abortus* and *B. melitensis* (1-6). It has been claimed that many strains may show biochemical characteristics of one "species", but serologic characteristics of another. For example, strains have been described that require no added CO₂, produce H₂S, grow in the presence of basic fuchsin or thionin, and agglutinate in the presence of monospecific *abortus* sera but not in monospecific *melitensis* sera (2). Such strains have been interpreted as "biochemically *melitensis*, except for H₂S, serologically *abortus*". Obviously, these strains could have originated from "typical" *abortus* strains by 2 mutational steps, *i.e.* a spontaneous change in CO₂-requirement and a spontaneous change in resistance to thionin. Marr and Wilson (7) have reported that the spontaneous rate of mutation from added CO₂-requirement to lack of such requirement in *B. abortus* is approximately 2×10^{-10} . In other words, any population containing 2×10^{-10} CO₂-requiring *B. abortus* cells is likely also to contain one mutant cell in which a spontaneous genetic change has caused the abolition of the added CO₂-requirement; all descendants of this cell would display similar properties. The rate at which determinants of other taxonomic characteristics of *Brucella*, such as dye-resistance and H₂S production, are likely to mutate never has been established. Also no data are available to indicate whether such mutants, once they have arisen, would be capable of competing successfully with their "typical" parent-type *in vivo*.

Methods. To investigate these points, the mutation rate to dye-resistance was determined with the help of the fluctuation test (8) for 4 strains of *B. suis* and 4 strains of *B. abortus*. The results indicate an average mutation rate of 6×10^{-10} (Table I). The

TABLE I. Rates of Mutation to Dye-Resistance in Smooth Strains of *Brucella abortus* and *Brucella suis*.

Parent strain	Mutation rate	Remarks
	$\times 10^{-10}$	
<i>B. abortus</i> , Weybridge 544-14-342 (CO ₂ -requiring)	9.9	Screened on 2-1 agar containing 1:30000 thionin
<i>B. abortus</i> , Spink 1301 (CO ₂ -requiring)	5.3	
<i>B. abortus</i> , 6232 (CO ₂ -requiring)	7.2	
<i>B. abortus</i> , NIH 313 (air-growing mutant)	6.6	
<i>B. suis</i> , NIH No. 6	6.8	Screened on 2-1 agar containing 1:50000 basic fuchsin
Weybridge P-2	9.1	
Spink 1330	6.9	
PSIII	5.2	
"	4.1	
"	4.4	
"	4.7	

ability of such mutants to compete with their parent type *in vivo* was then tested by infecting 188 guinea pigs subcutaneously with various known mixtures of dye-resistant mutant cells and their respective "typical" parental cells. All animals were sacrificed 4 weeks after infection and the proportion of dye-resistant cells in the recovered *Brucella* populations was determined by streaking ground and diluted samples of individual spleens and regional lymph nodes on both plain 2-1 agar and 2-1 agar containing dye (basic fuchsin in the case of *B. suis*, thionin in the case of *B. abortus*).

The results showed that most dye-resistant mutants can compete successfully with their "typical" parent type *in vivo* (Table II). Despite some variation among individual animals of each group, in general the proportion of aberrant cells recovered from the animals was similar to the proportion of such cells in the original inoculum. One exception was the basic fuchsin-resistant mutant isolated from *B. suis*, PSIII, which failed to compete successfully with its fuchsin-susceptible parent type and was practically eliminated following

TABLE II. Recovery of Mutant Strains of *Brucella* from Guinea Pigs following Mixed Inoculation with Their "Typical" Parent Type. All data are based on the recovery from five animals per group.

Type inoculated	Total No. of cells inoculated/animal	% recovery from spleen on dye-medium in comparison to recovery in plain medium	
		Avg	Range
<i>B. abortus</i> , 6232 = parent	8400	0*	
" , thionin-resistant	7000	100	
46% thionin-resistant + 54% parent	15400	40	25- 66
<i>B. suis</i> , NIH No. 6 = parent	790	0	
Idem	79	0	
<i>B. suis</i> , NIH No. 6, basic fuchsin-resistant	740	100	
50% NIH No. 6 basic fuchsin-resistant + 50% parent	1530	66	50- 77
95% " + 5% "	1564	95	65-119
<i>B. suis</i> , PSIII = parent	1100	2	<1- 3
" , basic fuchsin-resistant	1130	100	
50% PSIII basic fuchsin-resistant + 50% parent	2230	5	<1- 13
		% recovery from spleen in air in comparison to recovery under CO ₂	
<i>B. abortus</i> , Weybridge 544-14-342 (CO ₂ -requiring) = parent	8300	0	
" , air-growing mutant	6300	100	
43% air-growing mutant + 57% parent	14600	66	41-100

* Recovery from plain media was excellent in all cases listed in this column, which proved that each inoculum dose used constituted at least 1 ID₁₀₀. On dye-medium controls of certain resistant mutants did not yield exactly 100% recovery in comparison with plain medium (*e.g.*, 6232 thionin-resistant mutant consistently yielded 92% recovery on dye-medium compared with plain medium); in these cases experimental data have been adjusted on a comparative basis of 100% for control (*e.g.*, 92% = 100% or average of 37% observed in experiment of line 3 = 40% following appropriate adjustment).

mixed infection with parent and mutant cells. Data were also obtained for the *in vivo* competition between CO₂-requiring *B. abortus* cells and mutants capable of growing without added CO₂ (Table II). The latter appeared to have a particularly high selective value as indicated by their tendency to increase in proportion following mixed infection with their CO₂-requiring parent cells.

Efforts also have been made to determine whether it is possible to select from aberrant strains a number of mutant types that would more closely resemble standard strains. Table III shows 2 examples of such efforts. Two strains were obtained from Weybridge, England, which in their biochemical reactions resembled *B. abortus*, requiring CO₂, producing H₂S, and being capable of growing on basic fuchsin but not on thionin. However, according to the Weybridge investigators these 2 strains are serologically melitensis since they show a high degree of agglutination in mono-specific melitensis sera. It was relatively sim-

ple to isolate from these strains mutants which did not require added CO₂ for growth. From these mutants, in turn, other mutants were isolated which were able to grow well on thionin. As shown in the second and fourth line of Table III, these mutant strains now resemble typical melitensis strains except for H₂S production. So far it has not been possible to devise a screening technique which would permit the isolation of non H₂S-producing mutants from a population of H₂S-producing cells.

Discussion. Provided that the selective values observed for most of these mutants in guinea pigs reflect similar selective values in natural hosts, it does not appear too surprising that with increased attention towards the characterization of naturally occurring strains an increasing number of aberrant strains has been uncovered. The figures for spontaneous occurrence of mutants with aberrant diagnostic characteristics show that such types are likely to occur among any population of

TABLE III. Isolation of Mutants with "Typical" Characteristics from "Atypical" Strains.

Strain	Colony type	CO ₂ requirement	H ₂ S production on days				Growth in presence of:		Agglutination in monospecific sera;		Interpretation
			1	2	3	4	1:50000 B. fuchsini	1:30000 thionin	Abortus	Melitensis	
1301	S	+	+	+	+	+	+	—	++10	++160*	Biochemically abortus, serologically melitensis
Mutant from 1301	S	—	+	+	+	+	+	+			Now typical melitensis, except for H ₂ S
783	S	+	+	+	+	+	+	—	—	+++160*	Biochemically abortus, serologically melitensis
Mutant from 783	S	—	+	+	+	+	+	+			Now typical melitensis, except for H ₂ S

* Serological data from Weybridge; not rechecked because of lack of monospecific sera.

Brucella cells larger than 1×10^9 . Since many of these "aberrant" types apparently are not eliminated in competition with the more typical parent type, they should persist in any area where the incidence of brucellosis is relatively high. Furthermore, it can be assumed that under certain environmental conditions, perhaps in certain hosts, these aberrant types may attain an even higher selective value and thus replace their normal parent type. A survey of competition between these types in hosts other than the guinea pig should be of interest in this respect. Therefore, the *in vivo* experiments described above, recently were extended to studies with mice and the results obtained with 128 mice were identical to those previously obtained with guinea pigs. But of still greater interest would be a study of competition between aberrant and typical strains in their natural hosts, *i.e.*, cattle, swine, or goats.

Even though the reliability of some of the serological tests with monospecific sera has been questioned, any subsequent confirmation of lack of significance of the serological data reported by others would in no way affect the validity of the data and considerations presented here: accepted diagnostic characteristics are subject to spontaneous mutational changes and many of the resulting aberrant types possess considerable selective value *in vivo*. These observations, therefore, reempha-

size the dangers of utilizing characteristics subject to simple mutational changes for taxonomic purposes(9), and add weight to the argument that the now recognized species of *Brucella* represent varieties rather than valid species(4,5,10). However, from a practical standpoint it may remain useful to retain the so-called species of *B. abortus*, *B. suis* and *B. melitensis* as a reference standard, even though they may merely represent the most common biotypes of one species.

Summary. "Typical" *Brucella abortus* and *B. suis* strains were found to mutate spontaneously to dye-resistance (thionin and fuchsin, resp.) at an average rate of 6×10^{-10} . Most of the resistant mutants, and also mutants displaying altered CO₂-requirements, competed successfully with their typical parent type *in vivo*. The significance of naturally occurring aberrant *Brucella* strains and taxonomic problems have been discussed in the light of these findings.

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Isotopic Uric Acid in Gouty and Rheumatoid Arthritis Patients Treated with Probenecid and Phenylbutazone.* (21225)

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(Introduced by Fred R. Griffith, Jr.)

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There has been a clinical impression, not very well documented, that phenylbutazone (Butazolidin;† 3, 5-dioxo-1,2,-diphenyl-4-n-butylpyrazolidine) can lower the serum urate concentration markedly without causing a comparable excretion of "extra" uric acid. It was reported that both phenylbutazone and probenecid (Benemid;‡ p(di-n-propyl sulfa-myl)benzoic acid) caused a significant increase in urinary uric acid output in 2 gouty subjects but failed to do so in 3 patients with rheumatoid arthritis(1). Both drugs tended to cause a decrease in the serum urate levels although the effect was less regular in the gouty subjects than in the subjects with rheumatoid arthritis. In an attempt to elucidate the mechanism involved, isotope studies were carried out on 3 more gouty patients and 4 more patients with rheumatoid arthritis. The body uric acid pool size and turnover rate were determined during control and drug periods, and urinary uric acid excretion was determined by the isotope dilution method. The results indicate that within the experimental design used, both drugs cause statistically significant uricosuria only in subjects with increased body stores of uric acid.

Experimental. All patients were hospitalized and were kept under close observation. Their diet was uniformly low in purines and constant in calories, carbohydrate, fat and protein. The only medications allowed in addition to the test drugs were codeine (P.R.N. for pain) and non-barbiturate hypnotics. All urine was collected *ad libitum* and pooled into convenient samples. Uric acid was isolated from one aliquot of each pool for N¹⁵ determination and another aliquot was taken for uric acid determination by the isotope dilution method(2). The uric acid pool size and turnover rate were determined during the control period as previously described(2) and each patient was then placed on one of the test drugs for approximately one week. The uric acid pool size and turnover rate were determined in the second week of that period, during which the patient continued the same therapeutic regimen. The first drug was then discontinued and the second one given for approximately one week. During the second week of this therapeutic period the uric acid pool size and turnover rate were again determined. The daily dose schedule for probenecid was 3 tablets of 0.5 g each, and for phenylbutazone was 2 tablets of 200 mg each.

Results. The pool of miscible uric acid in normal individuals as determined in this and other laboratories(3) is usually not greater than 1200 mg (29 mMols U A nitrogen), and may be less in women. From Tables I and II it is apparent that only 2 patients, D. W., and A. B., were within normal limits. The other

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† Butazolidin was kindly supplied by Geigy Pharmaceuticals, Division of Geigy Co.

‡ Benemid was kindly supplied by Sharp and Dohme, Philadelphia, Pa.

TABLE I. Uric Acid Pool Size, Turnover Rate, Turnover, Excretion and Serum Concentration in 4 Rheumatoid Arthritis Patients after Treatment with Probenecid and Phenylbutazone.

Age, yr		Pool size, mMol UAN	Turnover rate, pools/day	Turnover, mMol UAN /day	Mean excretion, mMol UAN/day	Signifi- cance, p below†	Serum UA, mg %
D.W. ♀	74	C*	13.4	.94	12.6	9.95 ± .69†	%
		Pr	8.0	1.63	13.0	10.95 ± 2.56	not
		Ph	8.5	1.71	14.5	10.24 ± 1.47	"
A.B. ♀	59	C	15.2	1.04	15.9	10.33 ± 1.09	
		Ph	8.5	1.77	15.0	10.32 ± 1.57	"
		Pr	6.7	2.08	13.8	10.78 ± 1.59	"
L.F. ♀	58	C	36.9	.40	14.7	7.51 ± 2.32	
		Ph	36.4	.42	15.4	10.03 ± 2.74	2.5
		Pr	27.1	.59	16.0	12.14 ± 2.94	.5
J.S. ♂	67	C	39.0	.64	25.2	13.10 ± 1.83	
		Pr	20.6	1.05	21.6	15.31 ± 3.57	10
		Ph	30.0	.73	22.0	12.79 ± 2.35	not

* C = Control; Ph = Phenylbutazone; Pr = Probenecid.

† Stand. dev.

‡ † test(6).

5 patients had enlarged uric acid pools.

In the 2 patients, D. W., and A. B., both phenylbutazone and probenecid caused a marked reduction in the pool size and increase in the turnover rate. The turnover remained essentially constant. Yet despite this pronounced decrease in the pool size there was no significant increase in the urinary uric acid output. This is shown by the fact that the *t* tests between the means of the control and experimental periods were not significant.

The 3 gouty patients and one rheumatoid arthritis patient, L. F., demonstrated essentially the reverse of this situation, *i.e.*, a significant uricosuria with either drug and a less marked reduction in the pool size. The case of J. S. is equivocal and does not clearly fit into either category. Probenecid was more potent as a uricosuric drug than phenylbutazone. A *t* test between the means of the drug periods revealed significance below the 5% level in the rheumatoid arthritic patients L. F. and J. S. and on all gouty patients except D. F.

Perhaps the most striking feature of all these studies is that the turnover remains relatively constant in each individual even though the pool size, turnover rate, serum urate concentration, or urinary uric acid excretion may have been markedly affected by the drugs. On this basis one might reason that the rate of loss of uric acid through the kidney is determined by the serum urate level and the per-

cent of tubular resorption. Lowering the resorption rate(4,5) would immediately start to lower the serum urate level, and eventually equilibrium would set in at a lower serum urate and resorption level. If the body is not building up uric acid stores then the output at this new level must be the same as before, since the output of uric acid from the body must equal the rate of synthesis of uric acid in the body. The situation in the gouty individual is different in that he is building up uric acid stores and may be depositing uric acid in the solid phase. By drawing upon these stores he may keep his serum urate level elevated even though tubular resorption rate has decreased. Thus "extra" uric acid can be excreted and the miscible pool of uric acid will not necessarily be markedly affected. In this study the one rheumatoid arthritis patient (L. F.) that behaved like the gouty subjects did have an elevated serum urate level and an enlarged pool size.

The 2 cases, D. W. and A. B., demonstrate that uricosuric drugs can decrease the body uric acid pool size and serum urate concentration without causing statistically significant uricosuria. All the details of the mechanism cannot be elucidated by the present experimental design since in this procedure many days are grouped statistically to measure long range effects. To complement this data we need excretion data for the first few hours or

TABLE II. Uric Acid Pool Size, Turnover Rate, Turnover, Excretion and Serum Concentration in 3 Gouty Patients after Treatment with Probenecid and Phenylbutazone.

	Age, yr		Pool size, mMol UAN	Turnover rate, pools/day	Turnover, mMol UAN /day	Mean excretion, mMol UAN/day	Signifi- cance, p below†	Serum UA, mg %
							%	
D.F.	40	C*	60.8	.30	18.4	11.80 ± 3.48†		8.3
♂		Ph	55.5	.47	25.9	16.32 ± 4.99	.5	4.7-7.9
		Pr	62.7	.32	20.2	18.94 ± 5.07	1	5.8-8.0
M.B.	53	C	54.6	.41	22.6	7.71 ± .59		11.1
♂		Pr	41.2	.51	21.0	14.92 ± 2.02	.5	7.9
		Ph	40.5	.54	21.8	10.14 ± 1.84	.5	10.3-11.5
M.F.	37	C	56.5	.70	34.1	16.58 ± 2.52		10.4-11.7
♂		Ph	34.5	.92	31.6	13.05 ± 3.29	2.5	6.3- 9.7
		Pr	36.0	.98	35.1	30.72 ± 5.24	.5	5.3

* C = Control; Ph = Phenylbutazone; Pr = Probenecid.

† Stand. dev.

‡ t test(6).

days after the administration of these uricosuric drugs. Such studies are now under way in this laboratory and may explain what happens to the "extra" uric acid when the serum rate level decreases after drug administration.

Conclusion. Neither phenylbutazone nor probenecid caused a statistically significant uricosuria in 2 patients with rheumatoid arthritis, even though these drugs decreased the pool size of uric acid, increased the turnover rate, and decreased the serum urate level. In 3 gouty patients and 1 rheumatoid arthritis patient with an elevated serum urate concentration the drugs caused statistically significant uricosuria but variable effects on the pool size, turnover rate and serum urate concentration. In 1 patient with rheumatoid arthritis the results were equivocal. In 4 of these 5 cases the uricosuric effect of probenecid was statistically greater than that of phenylbutazone. It is postulated that the uricosuric drugs such as phenylbutazone and probenecid will

produce statistically significant uricosuria only in those cases in which increased body stores of uric acid are present. The present studies are based on experimental periods of 1-2 weeks for each regimen and the data do not indicate what happens in the first few hours of drug administration.

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Thromboplastic Activity of Leukemic White Cells.* (21226)

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This work was prompted by the observation that material aspirated from the bone marrow cavity of patients with acute monocytic leukemia clotted unusually rapidly. In contrast to the extreme thrombocytopenia exhibited by these patients, this finding suggested that a factor may be present in monocytes or their precursor cells with clot accelerating properties. The formation of thromboplastin in plasma is considered by most investigators to be due to the interaction of a platelet and several plasma factors. However, preformed thromboplastin is present in most tissues and cells(1). For this reason it appeared possible that thromboplastin might be found in monocytes, and preliminary experiments confirmed this assumption. In view, however, of marked differences in the chemical and enzymatic structure of leukocytes(2) this work was extended to the study of thromboplastic activity of various types of leukemic leukocytes, and striking differences were found.

Materials and methods. White cells were obtained from a total of 16 patients with various forms of leukemia (Table I), and a patient with periarteritis nodosa with a high eosinophile count. Only patients were studied in which white cell count exceeded 25,000/cu mm, to obtain a high yield of cells. The diagnosis of the type of leukemia was made after careful examination of the peripheral smear and bone marrow stained by the usual technics. All smears from patients with monocytic and myelo-monocytic leukemia were also examined after peroxidase staining. Thirty cc of blood were collected from each patient with 2-syringe technic. It was then transferred to Silicone-coated test tubes containing 1/10 volume of 1% Sequestrene-Na₂ solution (3). The tubes were centrifuged at 2,000

TABLE I. Thromboplastic Activity of Leukemic White Cells Extracts.

Diagnosis	No. of cases	Thromboplastic activity/million cells (expressed as γ of brain tissue)*
Acute granulocytic leukemia	3	62.5 (75.5-50.0)
Chronic granulocytic leukemia	3	62.5 (78.0-55.0)
Acute monocytic leukemia	1	280.0
Acute monomyelocytic leukemia	3	30.0 (25.5-42.0)
Acute lymphocytic leukemia	1	0
Chronic lymphocytic leukemia	4	0
Erythroleukemia	1	traces
Eosinophilia (periarteritis nodosa)	1	0

* (See Fig. 1.)

r.p.m. for 10 minutes, and supernatant plasma removed. The white cell layers were pipetted off, pooled into a glass test tube, and washed 3 times with normal saline. The white cells were finally resuspended in 3 ml of saline.† A white cell count was done on this preparation. The white cell preparation was then ground for 30 minutes in a mortar containing 5 g of sea sand. The resultant supernatant solution, after centrifugation at 2,000 r.p.m. for 15 minutes, appeared slightly turbid, but when examined microscopically contained no sediment (leukocyte extract). An unexpected difficulty was encountered in the preparation of extracts from lymphocytes, since these cells formed a viscous gel when washed in saline and could not be counted. The final extract was satisfactory. *Thromboplastic* activity of the leukocyte extract was determined by a modification of the method of Campbell and Stefanini for the platelet thromboplastic factor

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‡ Experiments were conducted using distilled water instead of saline solution, to facilitate lysis of the white cells and results similar to those described here were obtained.

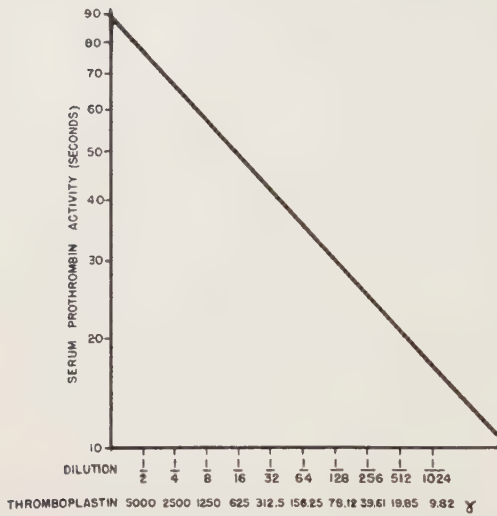


FIG. 1. Relationship of serum prothrombin activity of native human plasma to amount of rabbit brain thromboplastin.*

(4). Normal native human plasma was obtained by collecting blood from healthy individuals with the 2-syringe technic(5), transferring the blood into Silicone-coated test tubes which were then centrifuged at 3,000 r.p.m. at 4°C. All equipment was ice cold when used. While centrifugation proceeded, serial dilutions, in normal saline, of the leukocyte extracts were made. In a series of test tubes 0.9 ml of native plasma was added to 0.1 ml of each dilution of white cell extract. The mixture was rapidly transferred to a water bath at 37°C and allowed to clot. The clot-

* 10 mg of rabbit brain thromboplastin suspended in 1 ml of physiologic saline. Suspension incubated at 45° for 10 min. Tubes left standing 15 min. at 37°C to separate heavy particulate matter. It was arbitrarily assumed that supernatant contained entire thromboplastic activity; thus the presumptive weight in thromboplastin is indicated under each dilution. The serum prothrombin time of clotted native human plasma was then determined after addition of each dilution, one hr after completion of clotting (see text). The curve was used for calculation of thromboplastin activity of white cells. To translate thromboplastic activity of white cells into thromboplastic activity of brain tissue, the serum prothrombin time of native plasma containing the extract from 10 million cells was first determined (100000 cells/mm³). With use of graph it was observed to which concentration and weight of thromboplastin this result could be related. The figure obtained was then divided by ten, to express the thromboplastic activity of the leukocyte extract from 1 million cells.

ting time was recorded and the prothrombin time of serum was determined one hour after completion of clotting, according to a standard technic(6). Similar experiments were conducted using serial dilutions of rabbit brain thromboplastin instead of leukocyte extract, and a curve obtained showing effect of various concentrations of thromboplastin on prothrombin conversion of clotting native plasma, determined with the one-stage method (Fig. 1). The thromboplastin activity of leukocyte extract was then compared directly to the activity of a definite amount of rabbit brain thromboplastin, as described in legend of Fig. 1.

Results. A total of 17 white cell preparations were studied with regard to their thromboplastic activity, as outlined in Table I. Extracts of cells from patients with acute and chronic lymphocytes leukemia, marked eosinophilia (and from one preparation of pure erythrocytes) were found to have no effect on the clotting time or the serum prothrombin activity of human native plasma. Extracts from cells from patients with both acute and chronic granulocytic leukemia were found to accelerate clotting of native plasma, and increase the prothrombin consumption of native plasma (although they did not influence the extent of clot retraction). It was estimated by comparison with the thromboplastin dilution curve of Fig. 1, that one million granulocytes contained the activity of 62.5 γ of rabbit brain thromboplastin. Cells from patients with monomyelocytic leukemia were also found to be active (average thromboplastin activity: 30 γ/million cells). Finally, extract of cells from one patient with acute monocytic leukemia exhibited extremely high activity (thromboplastin activity: 280 γ/million cells). There appeared to be no appreciable relationship between degree of maturation of granulocytes and their thromboplastic activity, that was indistinguishable whether the cells were obtained from patients with the acute (predominantly immature) or the chronic (predominantly more mature cells) form of the disease. One patient with erythroleukemia (diGuglielmo's disease) was studied to investigate thromboplastic activity of immature red cells. This was very low, probably due to

contamination of the extract with substances from granulocytes, usually increased in number in the disease, and could not be separated from the normoblastic elements.

The ability of monocytic or granulocytic cells to correct the coagulation defect of hemophilia was also tested, since this disease is considered to be a classical example of thromboplastin deficiency. 0.1 ml of leukocyte extract from a patient with mono-myelocytic leukemia was mixed with 0.8 ml of fresh citrated hemophilic plasma and the mixture recalcified with 0.1 ml CaCl_2 0.2 M. The serum prothrombin activity of hemophilic plasma (determined by a one-stage technic) was reduced from 89% to 12% by addition of a leukocyte extract at dilution 1/4 with saline and to 43% by the addition of the same extract at dilution 1/16.

Discussion. There are only a few and contradictory results in the literature concerning the effect of white cells on the coagulation mechanism. Thus, Lenggehager (7) found that lysed leukocytes from normal and hemophilic blood shortened the clotting time of normal plasma. Martin and Roka (8) found that lysed white cells from patients with chronic granulocytic leukemia prolonged the recalcification time and the one-stage prothrombin time of normal plasma. These, however, were accelerated by lysed lymphocytes from patients with chronic lymphocytic leukemia.

Like the preceding observations, our results should be considered, qualitative or semiquantitative, since little is known of the solubility and stability of the thromboplastic agent or agents in the white cells. Even more arbitrary, but convenient, is the reference of the thromboplastic activity of white cells to that of rabbit brain thromboplastin, since one assumes the complete solution or suspension of the thromboplastin in the supernatant saline. The results obtained indicate unequivocal thromboplastin activity of some of the white cells and also point to basic differences between their various types. Monocytes and granulocytes supply thromboplastin; eosinophiles and lymphocytes do not. It appears probable that

the hypercoagulability of bone marrow material collected from patients with monocytic leukemia, may be explained by the release of thromboplastin from these cells. It should be pointed out, however, that the presence of thromboplastic material in leukocytes in no way improves the bleeding tendency of these patients. This remains closely dependent on the severity of the thrombocytopenia.

The possibility of correcting the hemostatic defect of hemophilia by addition of white cell extracts raises another interesting problem, perhaps related to the observation that administration of histamine to hemophiliacs may result in amelioration of the bleeding tendency and temporary shortening of the clotting time (9); one wonders whether the thromboplastin activity of granulocytes may not be linked to their high content in histamine (10).

Summary. Some types of white cells collected from leukemic patients (granulocytes, monocytes) but not others (lymphocytes, eosinophiles) release thromboplastic material. The extracted material is able to correct the coagulation defect of hemophilic plasma *in vitro*.

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Ultracentrifugal Analysis of Serum Lipoproteins in Nephrotic Syndrome of Rats.* (21227)

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The intravenous injection of nephrotoxic serum (N.T.S.) obtained from rabbits produces in rats a chronic renal disease in which hyperlipemia is always present. It simulates the nephrotic syndrome as it is seen in infants and children(1). The lipoprotein pattern has been studied in the human disease(2,3) and it seemed of value to determine whether similar changes occur in the rat.

Methods. Rats of the Long-Evans and Sprague-Dawley strains were fed dog chow† and water. Renal disease was induced according to the technic previously described(1) when they were 4-5 weeks of age. Animals were chosen that had a well-established disease for various lengths of time. Blood for serum protein, cholesterol and total lipid determinations was obtained by cardiac puncture 4-5 days before exsanguination when lipoprotein determinations were made. Serum lipoproteins were determined at a density of 1.21 by the method of Gofman(4) as modified by Lewis, Green and Page(5).

Results. Ultracentrifugation of lipoprotein concentrates of normal rat serum demonstrates 4 components. Dependent on decreasing density, their flotation rates are -S 1-10, 10-25, 25-40 and >70. Ninety to 120 mg of higher density -S 1-10 and 15-40 mg per 100 ml of -S 10-25 component are present, while there is only 15-30 mg of -S 25-40 component. The concentration of low density -S >70 material varies greatly (0 to 100 mg per 100 ml).

The lipoprotein concentration in serum of rats showing the nephrotic syndrome was much greater than that of normals (Table I). While the concentration of all fractions was sometimes increased the greatest increment was usually in the lower density fractions. The -S (40-70) component which is not usual-

ly present in measurable amount in normal rats was significantly increased. The -S 10-25 fraction was also often very large, and appeared heterogeneous as indicated by resolution of numerous small peaks.

There seemed to be no correlation between duration of the disease and the type or extent of lipoprotein changes. Rat no. 7 which had had the syndrome for 10 months showed the same type of change that rat no. 13 showed within 17 days. The lipoprotein changes of rats no. 3 and 4 with disease of longest duration and of lesser severity were less than in the other nephrotic animals.

In rats studied 17 days after injection of nephrotoxic serum, there was a total lipoprotein of 700 to 900 mg, or 15 to 35% of the total serum protein, which was 3 to 5 g per 100 ml. Rats which had had the disease 11 to 16 months had smaller concentration of lipoprotein 185 to 600 mg, or 3 to 12% of the total protein which was 4.9 to 6.0 g per 100 ml.

Discussion. While the absolute concentration of the lower density -S >70, 40-70 and 25-40 lipoproteins in the sera of nephrotic rats is not as great as in the nephrotic syndrome in the human being(3) the kind of change is similar. The normal concentration of -S 25-40 and 40-70 in human beings is much greater than in the rat so that the percentage increase in the two species may be comparable. Both species also show an increased concentration of -S 10-25 lipoprotein component.

The -S 25-40 fraction corresponds to the Sf 3-10 fraction determined at a density of 1.063, and the -S 40-70 to Sf 10-20. Gofman *et al.*(6) have observed an increased concentration of the Sf 10-20 class of lipoproteins in nephrotic sera and have emphasized the importance of this group of macromolecules in atherogenesis.

The change in the serum lipoproteins of rats injected with N.T.S. is very much greater than that observed in rats with acute renal

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† Friskies, Albers Milling Co., Kansas City, Mo.

TABLE I. Serum Lipoproteins, Determined at Density 1.21, Cholesterol, Total Lipids and Total Proteins of 13 Rats with the Nephrotic Syndrome.

Duration of disease	Severity of disease	Total protein, g/100 ml	Cholesterol, mg/100 ml	Total lipid, mg/100 ml	-S>70	40-70	25-40	10-25	1-10
mg/100 ml									
Control	—	5.2	60	360	99	—	26	20	114
"	—	5.2	75	440	39	—	20	38	91
16 mo	2+	5.96	75	1600	33	—	21	—	129 ⁺
13	3+	5.83	105	1460	42	21	33	106	152
12	4+	4.88	140	1680	164	19	71	141	223
11	4+	5.29	208	2360	77	24	75	141	182
10	4+	5.17	242	1880	282	38	59	106	152
7	4+	5.63	270	1900	398	—	60	86	315*
7	4+	3.42	550	3310	—§	282	282	—	101*
4 wk	4+	4.63	155	4720	170	—	34	57	199
17 days	4+	2.96	485	5880	552†	40	152	—	199*
17	4+	3.29	400	5240	>400	—	129†	71	168
17	4+	3.96	244	2650	282‡	16	47	164	164

* Spreading peak -S 1-15. † No clear resolution -S 25-70. ‡ -S 70-400; -S>400++++. § -S>400++++.

disease resulting from desoxycorticosterone acetate priming followed subsequently by renin injection(7). This may be due to differences in extensiveness of the lesions or duration of the disease.

Summary. Ultracentrifugal analyses at a density of 1.21 of serum lipoproteins in the nephrotic syndrome of rats induced by the intravenous injection of N.T.S. obtained from rabbits, showed greatly increased concentration of all lipoprotein components but chiefly in the lower density fractions. The amount of change was not directly correlated with the duration of the disease, but was generally comparable to its severity. The changes observed in the rat were similar in type to those in the nephrotic syndrome of children.

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Participation of Brown Fat in Pathogenesis of Experimental Poliomyelitis of Monkeys.* (21228)

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Progression studies have served to indicate some of the pathways by which peripherally administered poliomyelitis virus (Type 2) reaches the CNS in cortisone-treated hamsters

(1-3). Somatic lesions in two types of tissues were noted during the anteneural phase of the experimental disease. A myositis was demonstrable(4) as well as lesions in brown (hibernating) fat(5). High concentrations of virus were present in these foci. The following studies deal with the possible role of brown

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FIG. 1. Biopsy of brown fat resected from left axilla, 5 days after animal was inoculated intramuscularly into the left arm with Wisconsin strain (Type 1). Animal developed paralysis 3 days after biopsy. There are numerous islands of cellular necrosis and leucocytic infiltration. On subsequent intracerebral and peripheral passage the tissue produced typical poliomyelitis. H. & E. $\times 100$.

fat in pathogenesis of experimental poliomyelitis in the monkey.

Experimental. Young male cynomolgus monkeys were used in all experiments. Three strains of Type 1 poliomyelitis virus (MacMahon, Wisconsin, and Brabyn, isolated from human stool and typed by Dr. Robert Ward) were available for study. The inoculum consisted of centrifuged monkey cord emulsion, diluted 1:50 and injected in a volume of 1 cc. Cortisone was administered intramuscularly into hind limbs in daily dosage of 10 mg for a period of 7 days. Following the fourth injection of cortisone each strain was inoculated intramuscularly into the left arm of 2 monkeys. After a period of 5 days, biopsies of the ipsilateral axillary brown fat were taken from each animal under barbiturate anesthesia. A portion of each resected specimen was studied histologically and the remainder used for animal passage. For the latter purpose, the tissue was ground with sterile sand and mixed with saline solution to give a final dilution 1:50. The suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant filtered through sterile Whatman filter paper (No. 1). The filtrate contained no

gross particles and appeared opalescent. Animals infected with Wisconsin and MacMahon strains were sacrificed on the first day of paralysis and those inoculated with the Brabyn strain were killed the day following biopsy and prior to clinical paralysis. Complete autopsies were performed on all monkeys. Biopsied brown fat suspensions were passaged serially in cortisone-treated and untreated monkeys by intracerebral and intramuscular routes. Selected material was inoculated into tissue cultures of monkey renal epithelium(6). Axillary tissues other than brown fat obtained at autopsy were also inoculated into monkeys. The anatomy and histology of brown fat was studied in normal, cortisone-treated and infected monkeys.

Nature and distribution of brown fat in the cynomolgus monkey. Brown fat differs from ordinary adipose tissue in many respects. The former is lobulated, demarcated, confined to specified anatomic regions and dissimilar in biochemical constitution(7,8). Brown fat cells are smaller and contain centrally located nuclei. The cytoplasm contains small polyloculated lipid vacuoles separated by strands of eosinophilic matter. The tissue is

TABLE I. Serial Passages of Infected Brown Fat in Monkeys.

Animal No.	Inoculum	Site of inoculation	Cortisone	Day of onset of paralysis	Autopsy findings
I	Wisconsin monkey cord	I.M., left arm	+	8	Poliomyelitis
II			+	6	"
III	MacMahon monkey cord		+	7	"
IV			+	12	"
V	Brabyn monkey cord	I.C.	+	—	S† on 6th day. CNS normal
VI			+	—	" " 7th " " "
VII	Preparalytic axillary brown fat biopsy monkeys I, II		+	7	Poliomyelitis
VIII			+	5	"
IX	Postparalytic axillary brown fat (autopsy) monkeys III, IV	I.M., left arm	+	7	"
X	Preparalytic axillary brown fat biopsy monkeys V, VI		+	6	"
XI	Spinal cord, monkey VIII		+	5	"
XII	Tissue culture suspension infected with preparalytic axillary brown fat biopsy tissue from monkeys I, II		+	5	"
XIII		I.C.	+	6	"
XIV			—	6	"
XV			+	3	"
XVI			—	6	"
XVII		I.M., left arm	—	3	"
XVIII	Postparalytic axillary brown fat (autopsy) monkey VII		+	7	"
XIX			—	4	"
XX			+	6	"
XXI		I.M., left arm	—	6	"
XXII	Postparalytic axillary white fat (autopsy) monkey VII		+	—	S after 25 days. CNS normal
XXIII	Postparalytic axillary lymph node (autopsy) monkey VII		+	—	<i>Idem</i>
XXIV			+	—	"
XXV	Postparalytic axillary nerve trunk (autopsy) monkey VII		+	7	Poliomyelitis

* 10 mg/day for 7 days.

† S = Sacrificed.

endowed with a rich capillary circulation. The principal depot of simian brown fat was observed in the axillae in the form of adjoining lobules. A small amount of this tissue was also found between the posterior and lateral cervical musculature, as well as in the scapular region. Minute amounts were detected in the thoracic paravertebral regions and in the soft tissues encompassing the adrenal glands.

Results. Effect of cortisone upon brown fat of the cynomolgus monkey. Two cynomolgus monkeys were inoculated daily with 10 mg of cortisone for a period of 7 days and sacrificed 1 day after the last injection. Two control monkeys of similar age were sacrificed at the same time. Masses of brown fat from each of the 4 animals were dissected out, fixed in

buffered formalin solution and studied histologically. The polylocular configuration of brown fat cytoplasm was unusually prominent in cortisone-treated monkeys, and the cells visibly enlarged. Cell diameters were determined for 500 brown fat cells from each specimen. The mean diameter of brown fat cells of control animals was $16.4 \pm 0.15 \mu$, while that of the cortisone-treated animals was $25.3 \pm 0.64 \mu$. No inflammatory or degenerative features were visible in the microscopic sections of brown fat obtained from control and cortisone-treated monkeys.

Histological changes and viral proliferation in brown fat. Five to 7 days following viral inoculation into the upper limb muscles, multiple isolated lesions appeared in the ipso-

lateral axillary brown fat (Fig. 1). Lesions of approximately equal severity followed inoculation of all strains used. The primary changes consisted of fragmentation, beading and coarseness of the cytoplasmic strands. The degenerating nucleus was displaced peripherally. In early lesions small numbers of polymorphonuclear leukocytes were often present in the vicinity of necrotic cells. At a later phase of the process, inflammatory cells obscured the focus of necrosis. Perivascular inflammatory infiltration was seen in neighboring regions. In the terminal phase of the disease, lesions were also observed in the contralateral axillary and paravertebral deposits of brown fat.

As may be seen from Table I, axillary brown fat, resected during the preparalytic period from animals I and II infected with the Wisconsin strain, was inoculated into monkeys VII and VIII intramuscularly and intracerebrally. The animals developed paralysis on the 7th and 5th days, respectively. The cords from monkeys VII and VIII showed histologically typical poliomyelitis and the cord from monkey VIII induced the disease on intracerebral passage (monkey XI). Titration of the brown fat emulsion in tissue cultures revealed a cytopathogenic titer in the order of 5 logs. The tissue culture fluid was then inoculated into cortisone-treated and untreated monkeys, intramuscularly (XII-XIV) and intracerebrally (XV-XVII). All 6 monkeys developed paralysis within 6 days.

The contents of the axillary cavity of monkey VII were carefully removed and separated into component tissues. The resected brown fat was inoculated into cortisone-treated and untreated monkeys, intracerebrally (XX, XXI) and intramuscularly (XVIII, XIX). All 4 monkeys showed paralysis within 7 days. Axillary white fat was injected intramuscularly into cortisone-prepared monkey XXII. The animal failed to demonstrate any paralytic symptoms during the 25 day period of observation and showed no histological lesions. Axillary lymph nodes were inoculated intramuscularly into cortisone-treated monkeys XXIII and XXIV. No paralysis ensued and the CNS was histologically normal. Brachial plexus nerve trunks were passaged intramus-

cularly into cortisone-treated monkey XXV. This animal became paralyzed in 7 days and autopsy confirmed the presence of poliomyelitis.

Two monkeys (V, VI) inoculated intramuscularly with Brabyn strain were sacrificed 6 days following inoculation, prior to the development of paralysis. Lesions were confined to the ipsilateral axillary brown fat and the CNS showed no evidence of poliomyelitis. Brown fat was inoculated intracerebrally into a cortisone-treated monkey (X) which showed severe paralysis in 6 days. Autopsy showed typical poliomyelitis.

Two cortisone-treated monkeys (III and IV) infected intramuscularly with MacMahon strain were sacrificed the day of paralysis and the ipsilateral axillary brown fat was inoculated intracerebrally into monkey IX. This animal succumbed in 7 days and histologic sections of the brain stem and spinal cord showed extensive lesions of the anterior horns and motor segments of the medulla.

Discussion. In the cortisone-treated Syrian hamster, the preparalytic period is characterized by demonstrable somatic lesions, namely, myositis and necrosis of brown fat. The muscle lesions are morphologically indistinguishable from those induced by Coxsackie virus(9). Extraneural propagation of the poliomyelitis virus in muscles is probably unique to the hamster since we have been unable to demonstrate this phenomenon in mice or monkeys. There also occurs in the cortisone-treated hamster a marked proliferation of the virus in brown fat, commencing 2-3 days prior to neuraxial involvement and accompanied by severe necrobiosis. It is of interest to point out that most strains of Coxsackie virus show a similar lipotropic property in suckling mice, eventuating in lesions remarkably like those produced by Type 2 strains of poliomyelitis virus in the hamster(9).

While brown fat is more prominent in hibernating animals, it is nevertheless demonstrable in most mammalian species. In the monkey, the principal deposit is located in the axillary apices. Simian brown fat is essentially similar to rodent brown fat as determined by histologic and histochemical pro-

cedures. In addition, monkey brown fat undergoes hypertrophy under cortisone treatment in the same manner as does rodent brown fat(10).

Studies described in this paper indicate that following intramuscular inoculation into cortisone-treated monkeys, the virus is capable of proliferation in brown fat prior to CNS involvement, thus suggesting that under our experimental conditions the brown fat constitutes an extraneural site of viral multiplication. Detectable quantities of virus persist in this site during the paralytic stage, as well. Cortisone was used in subsequent passages to insure successful takes following parenteral introduction of the virus(11). It is pertinent to point out, however, that in subsequent passages the use of cortisone is not obligatory for production of steatitis, viral propagation in brown fat and CNS involvement. It remains to determine whether viral multiplication in brown fat on subsequent passages, in the absence of cortisone, was due to adaptation of the virus to this tissue.

It is of note that the widespread necrotic and inflammatory lesions in brown fat invariably coincide with viral propagation.

Summary. Following peripheral inoculation, poliomyelitis virus (Type I) proliferates in the brown fat of cortisone-treated monkeys during the preparalytic and paralytic stages of the disease. On subsequent peripheral pas-

sage of infected brown fat, the virus can be detected in the brown fat in the absence of cortisone treatment. Characteristic brown fat lesions are incidental to viral proliferation.

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In vitro Cultivation and Cytopathogenicity of Vesicular Exanthema Virus.* (21229)

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(Introduced by R. S. Muckenfuss.)

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The epizootic of vesicular exanthema of swine which spread over most of the United

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States in 1952-1953 brought to general attention the lack of information concerning the etiological agent. Although isolated as early as 1934(1), this virus has received comparatively little attention from virologists due, in part, to the consistent failure to propagate the agent with ease in any species except swine. The present report describes the first successful cultivation of vesicular exanthema virus

(VEV) by a tissue culture technic suitable for large scale production of infectious or antigenic material as well as for *in vitro* assay.

Materials and methods. *Virus.* The A type virus was that isolated during an outbreak near Fontana, Calif., in 1948, while the B type was obtained during a field outbreak in 1952 in the South San Francisco area of California. The type designation is based on the suggestion of Madin and Traum(2,3). *Preparation of tissue cultures.* Swine embryos ranging in age from 6-9 weeks, obtained from a local slaughterhouse, were removed aseptically from the uterus and washed in Hanks' solution containing penicillin, 500 units/ml, and streptomycin 0.5 mg/ml. Portions of skin, muscle, tongue and snout were minced with scalpels or scissors into fragments about 1 to 3 mm in size and washed repeatedly in Hanks' solution. The tissue mince was embedded in chicken or swine plasma in 15 x 150 mm pyrex tubes (4-6 fragments per tube) or in 4-ounce prescription bottles. Nutrient fluid consisted of swine serum (5%), swine embryo extract (5%) in synthetic mixture 199(4) to which penicillin and streptomycin were added in final concentrations of 100 units/ml and 50 γ /ml respectively. Two ml of nutrient fluid were added to each tube and 10 ml to each bottle. After incubation at 37°C for 48-96 hours with or without rolling, most of the tissue fragments showed considerable outgrowth of fibroblasts and epithelial type cells. *Infectivity tests.* Swine were inoculated with 1 ml volumes of tissue culture fluids by either the intradermal or intravenous routes. *Complement fixation tests.* The technic for the complement fixation test followed that described by Kabat and Mayer(5). The antigens were 1) ground infected tissue culture fragments suspended in the supernatant fluid and 2) the standard swine antigen prepared by grinding infected snout vesicle coverings. A control antigen of normal embryonic swine tissue was included. *Neutralization tests.* A suspension of infected tissue fragments in tissue culture fluid was diluted by 10-fold serial steps to 10^{-7} . Two ml quantities of the 10^{-2} through 10^{-7} dilutions were mixed with equal volumes of either normal or convalescent A or B type VEV swine serum diluted 1:4. Serum-virus mix-

tures were held at 37°C for 2 hours before inoculation into 48-hour hog embryo cultures. These cultures were examined daily for 96 hours and cytopathogenic changes noted. *Sterility tests.* Tests for bacteriological and mycological sterility of infected tissue culture fluids were carried out by inoculation of 0.5-1.0 ml into thioglycollate medium and onto Sabouraud's agar. Cultures were incubated at 37°C or at room temperature for 72-96 hours.

Results. Propagation of the B type virus in swine embryo cultures was initiated by adding 10 ml of an 0.1% suspension of infected swine snout epithelium in the nutrient fluid to a series of bottles containing embryonic tissue culture. No clear evidence of cytopathogenicity was obtained in the first passage. Approximately 96 hours after virus inoculation, tissue and fluids were harvested and ground. A 1 ml aliquot of this mixture was mixed with 9 ml of fresh nutrient fluid and added to each of 3 tissue cultures. Within 48-52 hours after inoculation, clear evidence of a cytopathogenic effect was apparent on low power examination. These changes were characterized by extensive destruction of the

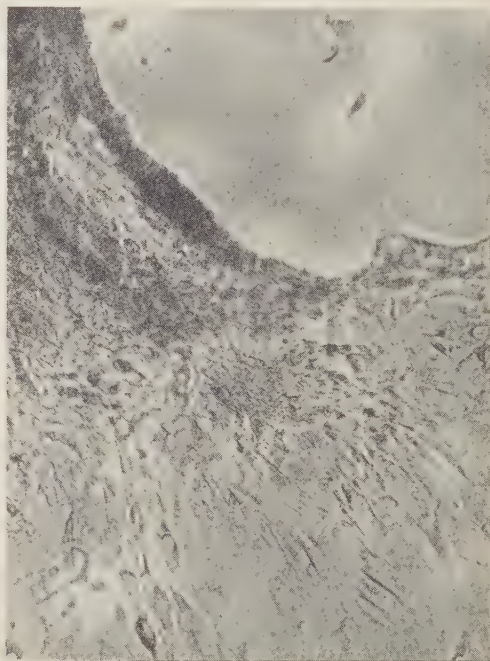


FIG. 1. Embryonic swine tissue culture not infected with VE virus. Approx $\times 100$.



FIG. 2. Embryonic swine tissue culture infected with VE virus. Approx $\times 100$.

cellular outgrowths around the tissue fragments. The normal fibroblastic and epithelial cells were replaced by rounded cells showing cytoplasmic degeneration and pyknosis of the nuclear elements. The contrast between the appearance of uninfected and infected cells may be seen by examining Fig. 1 and 2. Sixteen serial passages were made, each passage representing another 10-fold dilution of the preceding passage material. In each passage the same cytopathogenic changes were noted, in some of the later cultures as early as 18 hours after inoculation. No visible changes were produced when the tissue culture agent was inoculated into monkey kidney epithelial cultures or into HeLa cell(6) tube cultures. However, there was extensive and rapid cellular destruction when inoculations were made into swine kidney epithelial cultures. These results will be reported in detail at a later date. There was no evidence that the cytopathogenic changes were caused by bacterial or mycotic agents since none of the cultures for such organisms showed growth. Tests for possible rickettsial or pleuropneumonia-like organisms were not carried out.

Infectivity tests and challenges in swine.

Infectivity tests were carried out with suspensions from the 2nd, 8th and 16th tissue culture passages. The original virus-containing material had been diluted 10^{-5} , 10^{-11} and 10^{-10} respectively by serial transfer. Four swine were inoculated with material from the 2nd passage, 4 with material from the 8th and 6 with material from the 16th. All 14 animals developed severe clinical vesicular exanthema characterized by temperature elevation to 104° - 107° F within 24 hours, followed at 48 hours by extensive vesicle formation at the sites of inoculation on the snout and lips. Secondary vesicles on the feet were visible 72-96 hours after inoculation. These results show that an agent capable of producing typical vesicular exanthema in swine had been propagated through 16 tissue culture passages. The animals inoculated with the 8th passage material were allowed to convalesce for 30 days and were then challenged with known B type virus. This was completely negative and these animals were then inoculated with A type virus 21 days later. The 6 animals inoculated with the 16th passage material were divided into 2 equal groups, one challenged with B type virus, the other inoculated with A type virus 21 days later.

Complement fixation tests. Dilutions of serum from guinea pigs hyperimmunized with B type infected snout material were tested with a 10% suspension of the 9th tissue culture passage material, with 10% normal swine embryo, and with known B type antigen. In addition the tissue culture suspension was tested against type A hyperimmune guinea pig antiserum. The B type antiserum showed specific fixation titer of 1:16 when tested with tissue culture antigen, and a titer of 1:64 when tested with snout antigen. No fixation occurred with normal swine embryo antigen when mixed either with A or B type antisera. Nor did either the tissue culture antigen or the B type snout antigen show any fixation with the A type antiserum.

Neutralization tests. The cytopathogenic activity of infected fluids could be completely suppressed in tissue culture by a mixture of such fluids with serum from swine convalescent from B type VEV infection, but not by mixture with A type antiserum. Results of a

TABLE I. Challenge of Swine with Known Types of Vesicular Exanthema Virus following Infection with Tissue Culture Suspensions.

Infecting suspension	Challenge virus	
	Type A	Type B
8th passage B virus	4/4*	0/4*
16th " " "	3/3	0/3
Controls	3/3	2/2

* Numerator = No. of animals showing clinical evidence of vesicular exanthema. Denominator = Total No. of animals used.

tissue culture neutralization test are shown in Table II. Cytopathogenic effects were observed through the 10^{-5} dilution of culture material when it was mixed with normal swine serum, while none were observed when the dilutions of culture fluid were mixed with homologous B type antiserum. No neutralization occurred with the heterologous A type antiserum. The results of cross-challenge tests in swine, shown in Table I, and neutralization tests in tissue culture (Table II) indicate that the cytopathogenic agent propagated in swine embryo cultures was antigenically indistinguishable from B type VEV.

Discussion and conclusions. An agent capable of producing typical vesicular exanthema in swine has been propagated through 16 passages in a swine embryo tissue culture system. In the course of these passages the original material has been diluted to 10^{-19} , so that it is highly unlikely that any infective material from the original inoculum was carried

TABLE II. Neutralization of Tissue Culture Virus with A and B Vesicular Exanthema Swine Antisera.

Dilution of tissue culture virus	Normal serum	Convalescent serum	
		Type A	Type B
10^{-2}	4/4*	4/4*	0/4*
10^{-3}	3/3	4/4	0/4
10^{-4}	3/3	4/4	0/4
10^{-5}	3/3	0/4	0/4
10^{-6}	0/4	0/4	0/4
10^{-7}	0/4	0/4	0/4

* Numerator = No. of tubes showing cytopathogenic effects. Denominator = No. of tubes inoculated.

through all 16 passages. Multiplication of this agent in tissue culture was accompanied by extensive degeneration of epithelial cellular outgrowths which thus provided a simple means for *in vitro* assay of this virus. Considerable investigation is still required to determine the sensitivity and reliability of the system of tissue culture assay, whether in swine embryonic tissues or adult tissues such as kidney epithelium. Preliminary experiments have indicated that plaque formation on swine kidney epithelium using the method of Dulbecco(7) is feasible.

Evidence that the cytopathogenic agent was in fact the B type of the virus of vesicular exanthema was provided by the following observations: 1) The agent produced clinical vesicular exanthema in swine with subsequent immunity to challenge with known B type virus, without loss of susceptibility to infection with the A type virus. 2) Specific complement fixation was obtained when tissue culture agent was mixed with serum from guinea pigs hyperimmunized with B type virus, but not when mixed with serum from guinea pigs hyperimmunized with the A type. 3) Neutralization of the cytopathogenic effect was observed in tissue culture when the agent was mixed with B type immune swine serum but not when mixed with A type antiserum.

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Distribution of Lipids, Lipase and Alkaline Phosphatase in Renal Tubule of the Cat. (21230)

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A number of observers(1-5) have noted that unusually large amounts of lipids are found in the normal kidney tubules of the cat and Modell(3) reported the excretion of fat in the urine. Lipase and alkaline phosphatase have also been observed in the kidneys of various animals, *e.g.* Gomori(6) and Bourne(7). This investigation was undertaken to see if there was any correlation between the presence of these lipids and the distribution of lipase and alkaline phosphatase in the kidneys of young and old cats.

Material and Methods. Kidneys from 24 cats were used in these studies: 10 kittens or half grown cats up to 6 months of age and 34 cm in length from the occipital crest to the base of the tail; 14 adults of unknown ages and with lengths ranging from 37-47 cm. The kidneys were removed and weighed within 20 minutes of the beginning of anaesthesia by chloroform, Model(3,4). The average weight

of the kidneys of the younger cats was 4.2 g and of the old cats, 11.2 g. Thin pieces were cut from radial sections of the left kidney. These were fixed in ice cold acetone for the tests for alkaline phosphatase and lipase(6,8, 9) and formal-calcium for the study of lipids (10). Frozen sections were cut for the study of lipids and also for the enzymes. Sudan black B was used generally to color the fats and oil red O and hematoxylin were used for comparison. The unsaturated triglycerides were demonstrated by the presence of rose colored droplets after treatment with Nile blue sulphate(11).

Observations. There was no significant difference in the distribution of lipids as colored by oil soluble dyes between the kidneys of the kittens and half grown cats so observations on them have been grouped under one heading (Table I). The kidneys of older cats were unlike the younger mainly in the increase in

TABLE I. Distribution of Lipids, Lipase and Alkaline Phosphatase in Renal Tubule of the Cat.

	Lipids		Unsaturated triglycerides	Lipase		Alkaline phosphatase
	Young	Older		Young	Older	
Renal corpuscle	—	Small sparse	—	—	—	Variable reaction
Protruded epithelium	Fine drops	Many	Present or not	+	+	Brush border positive
Proximal convoluted a.	Fine many	Coarse loaded	Loaded	+	+	<i>Idem</i>
Medullary segment b. proximal	Coarse more than a	"	"	+	+	"
c. terminal	"	Less or absent	Less or absent	+	Less or absent	Reduced
Descending limb	Seanty	Seanty	—	—	—	—
Ascending limb	> desc.	> desc.	—	—	Slight	Positive free surface, first part
Distal convoluted	Seanty	Seanty	—	—	"	—
Collecting duct	Seanty absent in medulla		—	—	—	—

* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

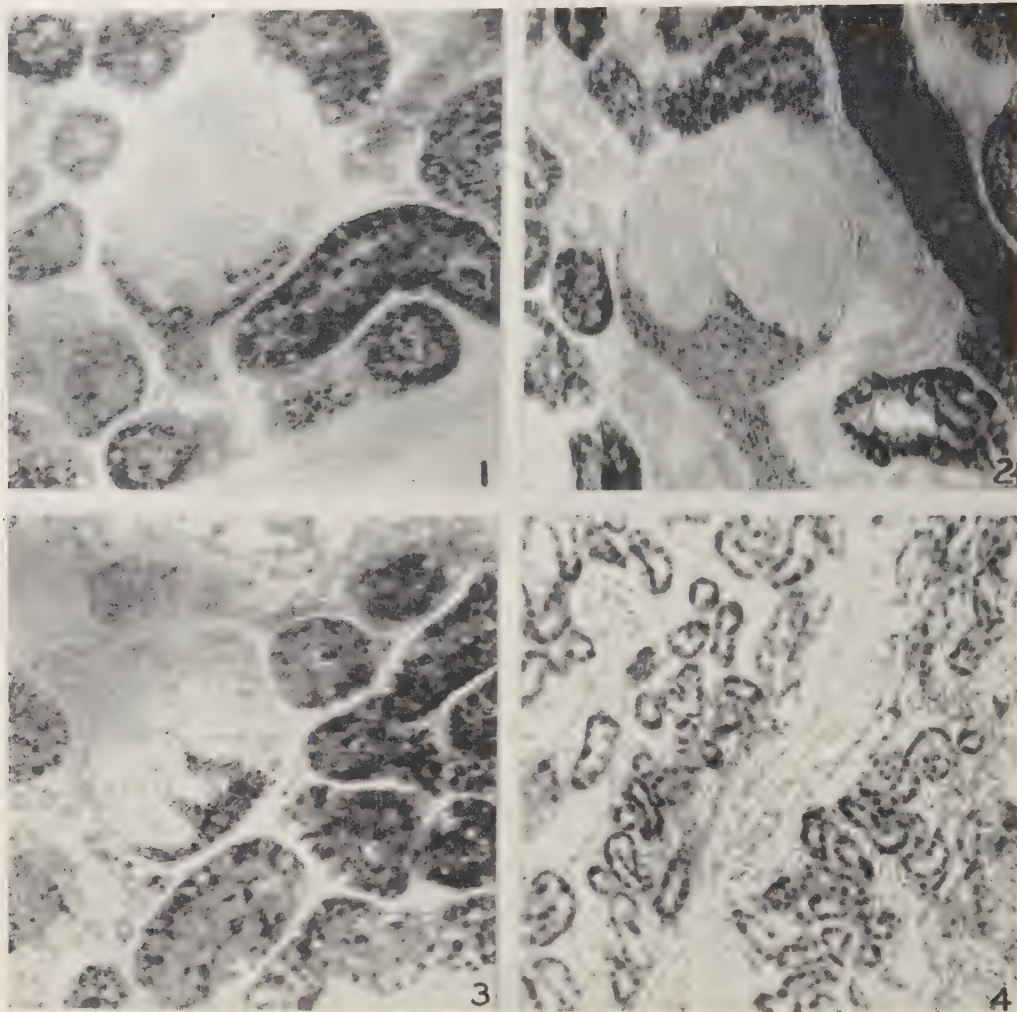


FIG. 1. Lipids; protruded and folded tubular epithelium in lumen of Bowman's capsule; proximal convoluted tubule. Sudan black B. $\times 250$.

FIG. 2. Lipids; protruded tubular epithelium, tangential section; proximal and distal convoluted tubules, ascending limb. Sudan black B. $\times 250$.

FIG. 3. Lipids; protruded epithelium outlining glomerular loops; proximal convoluted tubule, ascending limb. Sudan black B. $\times 250$.

FIG. 4. Lipase; pars convoluta. $\times 100$.

lipids in the proximal convoluted tubule and in the condition of the terminal portion of the medullary segment (Table I).

In 4 of the kidneys of the half grown cats and in all but one of the kidneys of the older cats, there were cells in the lumen of some of the Bowman's capsules characteristic of the proximal convoluted tubule. The folding of the epithelium within the capsular space is seen clearly in Fig. 1. Fig. 2 is a tangential section and Fig. 3 shows the characteristic

finger-like processes extending among the glomerular tufts. In all the corpuscles the parietal layer of Bowman's capsule could be distinguished outside of the protruded epithelium, a criterion of Mayer and Ottolenghi(12) for its diagnosis. These protruded cells and the neck epithelium contained lipid droplets but not as many nor as large as in the coiled portion of the tubule (Fig. 1, 2, 3).

A positive reaction for lipase in the proximal convoluted tubules (Fig. 4), their medullary

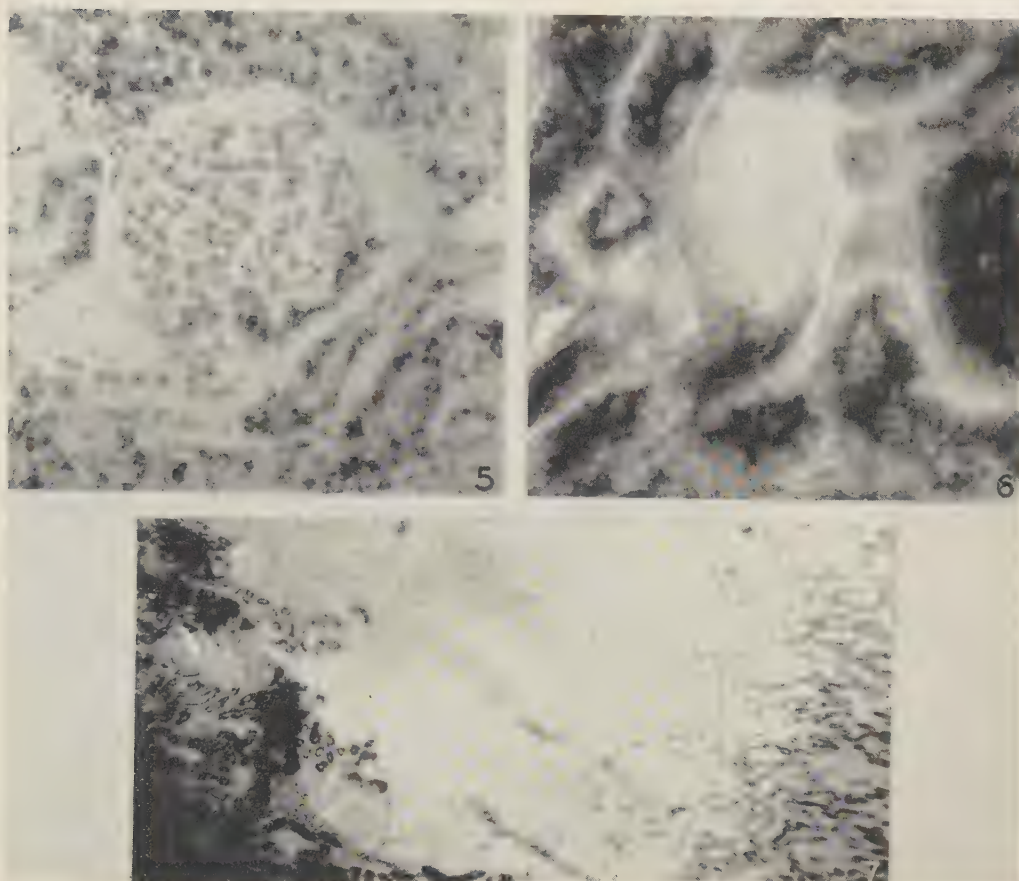


FIG. 5. Lipase; protruded epithelium, proximal convoluted tubule. $\times 250$.

FIG. 6. Alkaline phosphatase; brush border of protruded epithelium, neck and proximal convoluted tubule. $\times 250$.

FIG. 7. Alkaline phosphatase; brush border of proximal convoluted tubules on the left and free surface of cells of the first portion of the ascending limb on the right. $\times 35$.

segments and in the tubular epithelium within the capsular space (Fig. 5) correlated with the presence of fats in these regions (Table I).

The brush border of the cells of the proximal convoluted tubule, its medullary segment and the protruded epithelium gave a positive reaction for alkaline phosphatase in all of the kidneys (Fig. 6). In some sections it was the only positively reacting part of the cell. In others the nuclei were colored where the luminal surface gave a positive reaction. Besides the brush border there was a positive reaction of the free surface of the first part of the ascending limb of Henle's loop which formed a dark band across the medulla (Fig. 7).

Discussion. According to Modell(3), the

amount and distribution of lipids in the renal tubule of the cat do not vary with the state of nutrition, diet, or amount of intracellular fat present in other organs.

The most notable change in the distribution of lipids occurs with increasing age with a shift from fine droplets in the proximal convoluted tubules in young animals to much coarser and more numerous ones in the older cats, Smith(2). These droplets not only were colored in the oil soluble dyes but also gave a positive reaction for unsaturated triglycerides with the Nile blue sulphate method. There were no significant differences due to aging noted in the reactions for lipase and alkaline phosphatase. End portions of the medullary segments with some fat up to the descending

limb as well as those with none were present in all but one of the old kidneys. Because of the difference in fat content and also in cell contour in the terminal part, Foote and Grafflin(5,13) described 2 histologically different segments of the proximal convoluted tubule. The cells in the piece with no lipids were often edematous. In this condition of the medullary segment, triglycerides were not visible, there was little or no indication of lipase and the alkaline phosphatase reaction was often reduced to a fine, hardly discernible line. From this picture the assumption might be made that the terminal region of the proximal convoluted tubule is a vulnerable part of the kidney of the cat.

A discussion of the presence of epithelium protruded into the capsular space and a review of the literature can be found in the paper of Mayer and Ottolenghi(12), who concluded that the protrusions were found in mature renal corpuscles of 70% of the kidneys of young and adult dogs; there was no correlation with race, sex, age or history; the cause was unknown. In the present study these protrusions were found in 13 of the 14 kidneys of the older cats and in 4 of the 10 younger animals. Lehmann and Treuter quoted by Mayer and Ottolenghi(12) observed protrusions in 50% of the kidneys of cats. This tubular epithelium in the capsular space was found not only to contain lipids but also to react positively in the techniques for the demonstration of lipase and alkaline phosphatase. These observations confirm the view of Mayer and Ottolenghi(12) that the protruded epithelium

probably has a metabolism similar to that of the proximal convoluted tubule.

Summary. In the proximal convoluted tubule (its neck protruded epithelium and medullary segment) the presence of large amounts of lipids including triglycerides correlated with the positive reactions for lipase and alkaline phosphatase. If the terminal part of the medullary segment contained little or no fat, there was little or no indication of the presence of lipase or alkaline phosphatase. In those other regions of the tubule where lipids were scant or absent, there were slight or no reactions for lipase and none for alkaline phosphatase with the exception of the free surface of the first part of the ascending limb of the loop of Henle which gave a positive reaction. Protrusions of tubular epithelium into the capsular space were common in the kidneys of old cats.

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Production of Antisera Against Plasma Lipoprotein Fraction.* (21231)

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Interest in the measurement of plasma lipoproteins has recently increased because of the findings that atherosclerotic patients often have elevated contents of these substances. The development of a serological method for this assay would be anticipated because of the protein nature of the molecule. A previous attempt to produce an antisera against lipoproteins involved the injection of dog lipoprotein fraction into rabbits(1). Antisera reacting with the plasma of dogs were developed but no evidence was presented to demonstrate rigorously a true antigen-antibody reaction specific for lipoproteins. The present study represents an attempt to furnish proof of an antigen-antibody reaction specific for lipoproteins.

Methods. Lipoprotein antigen was obtained from the sera of rabbits fed 1% cholesterol and 3% corn oil in addition to regular rations for 30 days. The serum was spun at 30000 rpm in the Spinco model LH preparative ultracentrifuge for 24 hours. The top 2/9 was removed, diluted from 10 to 20 times with saline and injected intravenously into young chickens at intervals over a month's period. The injection schedule suggested by Kabat and Meyer(2) was used. Four to 7 days following the last injection, blood was removed from the chickens and serum or plasma separated. *Antiserum* against human lipoproteins was prepared in the chicken in the same manner. Sera obtained from atherosclerotic outpatients were used as the source of lipoprotein antigen. Titer of antisera was measured by the interfacial precipitin test. Undiluted antisera, 0.1 cc, was placed in the bottom of a 5 mm diameter test tube and 0.1 cc of appropriately diluted plasma containing lipoproteins was layered over it. The development of a white ring at the interfacial zone constituted a positive precipitin test. Tubes

TABLE I. Comparison of Titers Obtained by Mixing Sera of Various Animals (Antigen) with Each of 2 Chicken Antisera.

Antigen	Antisera	
	Chicken anti-rabbit sera	Chicken anti-human sera
Rabbit—		
Fat + cholesterol fed	10000-50000	1600
Normal	400- 1600	N at 400
Human—		
Atherosclerotic	800- 1600	800-3200
Normal	400	400
Chicken	N*	N
Rat	400	—
Sheep	40	—
Cow	80	—
Horse	160	—

* N = Negative.

were usually read after 60 minutes. Control tubes containing saline and normal chicken plasma were included. More quantitative information as to the concentration of antigen was determined by measuring the total nitrogen content of the washed precipitate. In this procedure the colorimetric method of Folin and Ciocalteu(2) was used.

Results. When sera obtained from high fat and cholesterol fed rabbits was titrated against antisera removed from chickens immunized against rabbit lipoprotein fraction, precipitin titers up to 50,000 were observed. Normal rabbit sera, however, yielded titers up to 1600. Chickens immunized against human lipoproteins yielded antisera capable of differentiating the atherosclerotic sera from that obtained from normal persons. Results of titrations of various animal sera with anti-human and anti-rabbit sera are presented in Table I in summary form. It will be noted that a certain amount of cross-species activity was present. Since the sera of rabbits fed high fat and cholesterol contained from 5 to 10 times the lipoprotein concentration found in the human atherosclerotic patient, it was not surprising that values obtained with rabbit antigen fraction exceeded those obtained with human.

In order to determine the degree of speci-

* Preliminary account presented at Meeting of American Physiological Society, Apr. 1954. (*Fed. Proc.*, 1954, v13, 60).

TABLE II. A Positive Correlation (0.69) between the Plasma Lipoprotein Values of Rabbits Measured by Precipitin Test and by Ultracentrifugal Analysis.

Rabbit	Precipitin ppt. mg N	Ultracentrifugal S _r 0-400 mg %
Normal	.03	81
	.12	54
	.14	92
Fat and cholesterol fed	.29	1571
	.33	2181
	.40	2090
	.44	3167
	.45	2908
	.45	3345
	.49	3829
	.49	3855
	.53	1557
	.56	5412

city of the antisera for lipoproteins, the total lipoprotein values (S_r0-400) obtained by ultracentrifugal analysis(3) of rabbit sera were compared with the total nitrogen of the precipitate obtained when the same rabbit sera reacted with chicken antisera. Serum obtained from 3 normal rabbits and 10 rabbits fed 1% cholesterol and 3% corn oil supplements was used. In practice 1 cc of the rabbit serum diluted from 1/20 to 1/80 with saline was mixed with an equal volume of undiluted chicken antiserum. After 1 hour at 38°C, the tubes were placed for about 16 hours in the icebox at 4°C. The washed precipitates were then analyzed for total nitrogen.

The results of this study are shown in Table II. A statistically significant positive correlation coefficient of 0.69 existed between measurements made ultracentrifugally and those measured serologically. A similar correlation was made using the sera obtained from 16 human subjects, 8 of which were diagnosed as atherosclerosis patients. The results in Table III showed a positive correlation of 0.92 which was highly significant.

A convenient method for the demonstration of the antigen-antibody reaction is that described by Ouchterlony(4-6) using the gel diffusion technic. With this method, in Fig. 3, a reaction of identity was established between the lipoproteins of normal human sera and those of the sera obtained from atherosclerotic patients. Although it has been

demonstrated that the lipoproteins are quantitatively increased in concentration in the sera of atherosclerotic patients, the gel diffusion plates prove them to be qualitatively identical by serological criteria.

Human lipoproteins of the S_r6 class were isolated in purified state by Dr. Ray A. Brown of these Laboratories. This preparation gave strong precipitin tests with chicken anti-human antisera. Used in the gel diffusion plate S_r6 lipoproteins gave the reaction of identity with the lipoproteins contained in unfractionated human sera.

In an attempt to eliminate certain non-specific and non-antigenic reactions, cholesterol and the cephalin-cholesterol reagent were added to immunized and normal chicken sera. Cholesterol alone had no visible effect while the cephalin-cholesterol reagent (Wilson Laboratories) produced an equal precipitation in both immunized and normal chicken sera.

Discussion. In order to establish the reaction described in this study as a true antigen-antibody precipitin reaction specific for plasma lipoprotein, the following observations are listed: 1) Rabbit and human sera containing lipoproteins will not generally react with normal chicken sera. Chickens must first be immunized with the appropriate foreign lipoprotein fraction. 2) The activity of the unfractionated chicken antisera was present in the redissolved precipitate obtained after 1/2

TABLE III. A Positive Correlation (0.92) between Human Plasma Lipoprotein Values Obtained by Precipitin Measurement and Ultracentrifugal Analysis. Sixteen subjects.

Precipitin ppt. mg N	Ultracentrifugal S _r 0-400 mg %
.16	202
.17	270
.18	362
.21	389
.21	544
.22	405
.22	594
.24	445
.24	598
.25	615
.27	661
.30	755
.31	770
.39	648
.40	837
.51	1357

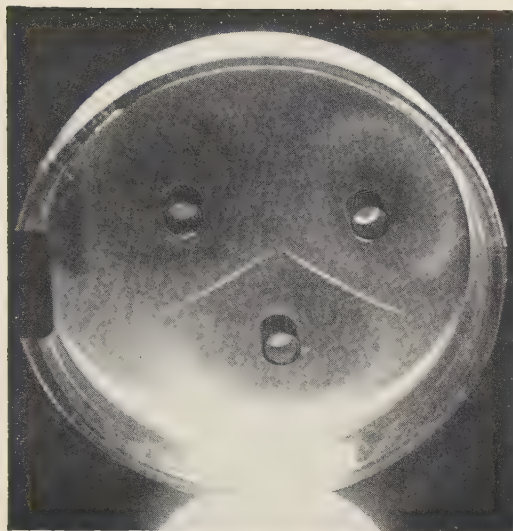


FIG. 1. Gel diffusion plates. *Left cup*: Normal human plasma. *Right cup*: Atherosclerotic plasma. *Center cup*: Chicken anti-human lipoprotein serum. The smooth blending of the lines of individual precipitates indicates the reaction of identity.

saturation with ammonium sulfate. 3) Chicken anti-human sera reacts with purified S_{r6} lipoproteins. The reaction of identity (Ouchterlony) was given between purified lipoprotein and those present in atherosclerotic plasma. 4) A high degree of positive correlation existed between the total lipoproteins

as measured by the ultracentrifuge and the quantity of precipitate produced by chicken antisera.

Summary. The injection of a lipoprotein fraction from the sera of cholesterol-fed rabbits or human atherosclerotic patients into chickens brought about the formation of antibodies specific for either rabbit or human lipoproteins. A positive correlation existed between the amount of precipitate produced by mixing antisera with sera containing lipoproteins and the lipoprotein content measured ultracentrifugally.

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Inhibitory Effects of Normal Guinea Pig Serum and Immune Rabbit Serum on Transplanted AKR Lymphomas.* (21232)

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When normal guinea pig serum is given intraperitoneally in relatively large amounts to C3H mice carrying the Gardner 6C3HED lymphosarcoma or to A mice carrying Lorenz's Lymphoma II, the growths regularly regress, as a recent study has shown(1); furthermore, the potency of the normal guinea pig serum

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against 6C3HED lymphoma cells *in vivo* is considerably enhanced when a small quantity of an immune serum, made by injecting the lymphoma cells into rabbits, is given together with it(1). In the work now to be reported normal guinea pig serum and immune rabbit serum, separately and in combination, have been tested for inhibitory effects *in vivo* against the cells of 8 transplanted lymphomas of AKR mice. As will be shown, the findings with the AKR lymphomas differ in at least one respect from those obtained with the C3H

and A lymphomas; this difference may have significance in light of the fact that lymphomas occur spontaneously in a high proportion of untreated AKR mice while being rare in animals of the C3H and A breeds.

Materials and Methods. Seven of the 8 lymphomas here employed were originally transplanted in this laboratory. One of the growths (Lymphoma L-1) was transferred initially from the enlarged nodes of an AKR mouse supplied by Dr. Lloyd Law of the National Cancer Institute, and 6 others from AKR/JAX mice with autochthonous lymphomas procured from the Jackson Memorial Laboratory. The provenance of the 8th growth (Lymphoma E-1) will be given further on. All the growths were carried in AKR/JAX mice, suspensions containing known numbers of cells being implanted in the groins of each host animal according to a standardized technique(1). As a rule palpable and visible tumors appeared some 6-15 days following implantation of the lymphoma cells; the growths regularly enlarged progressively thereafter until death of the animals, usually within 18 to 30 days. The normal guinea pig serum was procured by bleeding 12 to 36 normal, adult, market-bought guinea pigs without anesthesia, and allowing the blood to clot in paraffin-lined tubes. The serum specimens were harvested the same day, pooled, and stored in the deep freeze at approximately -22°C until used, usually within 6 weeks. The immune rabbit serum was prepared as follows: approximately 6 g of tumor tissue from Lymphoma L-1 was pressed through a monel metal sieve and suspended in 25 cc of 0.9% NaCl; the suspension was mixed with 25 cc Falba, 25 cc Bayol F, and 165 mg dried *Mycobacterium butyricum*, the mixture then being emulsified in a Waring blender. 2 cc of the creamy emulsion was injected intramuscularly into 6 sites in each of 6 large, market-bought, hybrid rabbits. The rabbits, all with large granulomas at the injection sites, were bled from the heart (approximately 50 cc) on the 21st day, and again 10 and 20 days later, the serum specimens being pooled and stored in the deep freeze.

The tests for *inhibition in vivo* were made as follows: One to 6 million lymphoma cells,

suspended in 0.5 cc of buffered Ringer's solution, were injected into the subcutaneous tissues of the groins of 8 to 24 or more AKR/JAX mice weighing 18-22 g each. The mice were then divided into groups, 3 or 4 animals in each. One or more of the groups was kept untreated as controls. The mice of 2 or 3 or more groups were given various amounts of normal guinea pig serum intraperitoneally, those of one group always receiving 2.0 cc of whole serum approximately 1 hour after implantation with the tumor cells; often the animals of another group were given 2.0 cc of the normal guinea pig serum 1 hour after the implantations and again on the 2nd and 3rd days of the experiment, though sometimes a single injection of a smaller amount of serum was given, or repeated injections of 2.0 cc of 3:1 "fan-concentrated" guinea pig serum(1). The rabbit immune serum was usually given to at least 2 groups of the implanted mice, usually in doses of 0.5 cc and 0.1 cc, respectively, sufficient 0.9% NaCl being added to make the volume of each injection up to 2 cc. Mixtures containing 1.5 cc normal guinea pig serum plus 0.5 cc immune rabbit serum, or 1.9 cc of normal guinea pig serum plus 0.1 cc immune rabbit serum, were injected into each of the animals in additional groups, again approximately 1 hour after the implantations. The immune rabbit serum was usually used unheated; in several experiments, however, its effectiveness was found to remain undiminished when it had been diluted 1:2 and heated at 65°C for 30 minutes. For control purposes, normal rabbit serum was used in a number of experiments; it had no effect on the AKR lymphomas when given alone or in combination with normal guinea pig serum. Except for some transitory sluggishness and tachypnoea, the injections as a rule did not bring about any signs of discomfort, the mice remaining lively throughout the periods of observation. Palpable tumors usually appeared at the implanted sites within 7 to 10 days in the control animals. Beginning at this time records were made every day or every other day of the state of each animal, with chartings of the tumors or a note that growths were absent.

Results. In several comparative tests done

TABLE I. Inhibitory Effects *in Vivo* of Normal Guinea Pig Serum and Immune Rabbit Serum, Separately and in Combination, on the Cells of Eight Transplanted Lymphomas of AKR Mice.

Summary of results of tests for inhibition <i>in vivo</i>				
8 lymphomas employed	Serial transfers tested	With normal guinea pig serum	With immune rabbit serum	With normal guinea pig serum plus immune rabbit serum
1-3	2nd, 3rd	0	?	?
1-6	1st	0	nt	?
2-2	1st, 2nd	0	++	++
1-4	2nd, 3rd	0	++	++++
1-7	2nd, 3rd	?	+++	++++
1-5	2nd, 3rd	?	+++	++++
L-1	1st, 2nd, 6th, 7th	?	+++	++++
E-1*	26th, 30th	++	nt	++++

See text for details of materials and methods and for results of concurrent tests with Gardner lymphosarcoma in C3H mice.

0 = No inhibition—growths in 3 or 4 test mice comparable in time of appearance, size, and course to those in untreated control mice.

? = Dubious inhibition; sometimes definite but slight inhibition in one experiment with little or no inhibition in a subsequent test.

++ = Moderate inhibition—growths appeared 2 to 5 days later than in controls, remained smaller throughout period of observation.

+++ = Marked inhibition—growths appeared 4 to 8 days later than in controls, remained smaller throughout period of observation.

++++ = Complete inhibition—no growths in test mice during 44-60 days; growths palpable in control mice after 7 to 10 days—these enlarged steadily, bringing about death with widespread lymphomatosis within 18-30 days.

nt = not tested.

* Lymphoma E-1 was transplanted for 25 generations during 2 years in laboratory-bred AKR mice of the line in which it had originated; it was then transferred to AKR/JAX mice for the tests here reported.

along with the experiments of the present work, inhibition of the Gardner 6C3HED lymphoma cells was regularly marked (+++) or complete (++++), when 2 cc of normal guinea pig serum was given intraperitoneally 1 hour after the subcutaneous implantation of 4 to 6 million 6C3HED lymphoma cells in C3H mice, and it was always complete (++++), when 2 cc was given on the day of implantation and on the 2 following days as well. Table I provides a summary of the results of the tests for inhibition of the 8 AKR lymphomas *in vivo*. The findings as a whole (Table I) show that normal guinea pig serum was far less effective against the cells of transplanted AKR lymphomas *in vivo* than it was against the cells of the C3H and A lymphomas previously tested(1) and confirmed in the case of the 6C3HED lymphoma in the present work. Indeed under the conditions here employed normal guinea pig serum did not notably inhibit the cells of any of the 7 recently transplanted

AKR lymphomas against which it was tested, this being generally true when 2.0 cc of the serum was given 1 hour after implantation with the lymphoma cells and again on each of the following 2 days. Additional tests showed that 2 cc of 3:1 "fan-concentrated" guinea pig serum(1), given to test mice on the day of implantation and again on each of the 2 succeeding days, had no effect on the later transfers of the cells of Lymphomas 1-7 and L-1. Furthermore, the normal guinea pig serum had only limited inhibitory effects *in vivo* on the cells of an AKR lymphoma (E-1) that had recently been transferred to a new line of AKR mice after 25 serial transplantations during approximately 2 years in the laboratory-bred AKR mice in which it had originated. It is possible that intrinsic differences in the neoplastic cells of the AKR and C3H lymphomas might account for the diverse results obtained in the inhibition tests with the AKR and C3H lymphomas. It is also possible that the mice of the 2 breeds

might react in significantly different ways following the injection of normal guinea pig serum intraperitoneally. And since the 6C3HED cells are killed *in vivo* when guinea pig serum is given to mice carrying them, but not *in vitro* when the cells are held in contact with whole guinea pig serum during several hours at 37°C—the effect *in vivo* obviously depending upon some reaction in which the animal host and the guinea pig serum both participate(1)—, the latter possibility has added interest. A third possible explanation for the findings will receive mention further on, after consideration has been given to the effects of normal guinea pig serum on the AKR lymphoma E-1.

The AKR lymphoma E-1 was moderately inhibited in the test mice given 2 cc of normal guinea pig serum one hour after the implantations, as Table I indicates; in other tests it was markedly but incompletely inhibited (+++) when 2 cc of the serum was given one hour after the implantations and repeated on each of the 2 succeeding days. In additional experiments, subcutaneous E-1 lymphomas measuring up to 1.5 cm across, resulting from implantations made 6-9 days previously, dwindled markedly in size within 24-48 hours following the injection of 2 cc of 3:1 "fan-concentrated" guinea pig serum intraperitoneally into mice carrying them, while gross and microscopic studies of the tissues of several animals of this experiment made it plain that visceral infiltrations of this lymphoma—e.g., in thymus, lungs, liver, spleen, and kidneys—had either failed to develop or regressed following injection of the concentrated serum. This growth had been transplanted longer than any of the other AKR lymphomas, and the fact may have significance for the interpretation of the results, though in this relation it may also be significant that the lymphoma L-1 was not inhibited after 6 and 7 serial transfers (Table I). Lymphoma E-1 differed from the rest of the AKR growths in another way as well: it had been transferred during approximately 2 years in AKR mice of the line in which it had originated (mice bred in the laboratories of Dr. Ralph Engle of this Medical Center) and then was transferred to AKR/JAX mice for

the experiments here reported. All the other lymphomas had originated in AKR/JAX mice and were regularly transferred in hosts of that line. These facts deserve consideration in relation to the fact that the 2 lymphomas previously found to be markedly inhibited by normal guinea pig serum *in vivo*—Gardner's lymphoma 6C3HED and Lorenz's lymphoma II (1)—had both been repeatedly transplanted during several years' time and eventually were tested in hosts manifesting some resistance against them. Considered together the findings lend weight to the supposition that isoantibodies might be provided by the hosts which act together with the intraperitoneally injected guinea pig serum in bringing about regression of subcutaneous lymphomas *in vivo*(1).

An immune rabbit serum—made by injecting the cells of one of the AKR lymphomas (L-1) together with Freund's adjuvants into rabbits, as previously described—, when injected in 0.5 cc amounts intraperitoneally into AKR mice one hour after implantation, proved moderately or markedly inhibitory for the cells of 5 of the 6 recently transplanted AKR lymphomas against which it was tested, its effects in the case of the remaining growth being dubious (Table I). The findings have interest in relation to the observation made some years ago by Nettleship that the Murphy-Sturm lymphosarcoma of rats was to some extent inhibited *in vivo* by large amounts of an immune serum made by injecting extracts of the rat lymphosarcoma cells into rabbits (2), and to the more recent observation of Nungester and Fisher that the 6C3HED lymphoma was likewise inhibited *in vivo* by an immune serum made in rabbits(3). Furthermore, mixtures containing 1.5 cc of normal guinea pig serum and 0.5 cc immune rabbit serum, given intraperitoneally 1 hour after implantation of AKR mice with the various lymphoma cells, proved markedly inhibitory *in vivo* for the cells of 5 of the 8 transplanted growths, the effects of the mixtures in these instances being often complete and usually greater than when either material was injected alone (Table I); in additional tests, mixtures containing 1.9 cc of normal guinea pig serum plus 0.1 cc of the immune serum were often markedly inhibitory, whereas mixtures con-

taining 1.9 cc of Ringer's solution and 0.1 cc of immune serum had only slight inhibitory potency or none at all. The observations may have a relation to a finding already reported—namely that the effectiveness of normal guinea pig serum against 6C3HED lymphoma cells *in vivo* is enhanced upon admixture with an immune serum made in rabbits with the 6C3HED lymphoma cells as antigen(1)—and it may have other implications also. The possibility that one or another of the components of complement may participate in the reactions responsible for these *in vivo* effects has been discussed in a previous paper(1). The question is now being tested whether immune serum alone, or mixtures of normal guinea pig serum and immune rabbit serum, will bring about regression of established AKR lymphomas *in vivo*.

Summary. In the experiments here given, as in those previously reported, normal guinea pig serum, given in amounts of 1 or 2 cc intraperitoneally to C3H mice one hour following the implantation of 2 million 6C3HED lymphoma cells in their subcutaneous tissues, always markedly inhibited growth of the lymphoma cells, and when 2 cc of the serum was given on the day of implantation and again on the 2 succeeding days the inhibition

was regularly complete. In striking contrast, normal guinea pig serum, even when given repeatedly in the largest feasible amounts, had little or no inhibitory effect *in vivo* on the cells of 7 AKR lymphomas recently transplanted in AKR mice of the line in which they had originated, and it manifested only limited effectiveness against the cells of a single AKR lymphoma that had been transplanted during about 2 years in laboratory-bred mice of the line in which it had originated with later transfer to AKR mice of another inbred line. An immune serum, prepared by injecting rabbits with the lymphoma cells of one of the AKR growths together with Freund's adjuvants, proved moderately to markedly inhibitory for the AKR lymphoma cells when injected intraperitoneally into mice one hour after implantation of the cells in the subcutaneous tissues, and mixtures of the immune serum with normal guinea pig serum were often completely inhibitory. The significance of the findings is briefly discussed.

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Secretion of 17-Hydroxycorticosterone by Adrenal of Hypophysectomized Dog: Effect of ACTH.* (21233)

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(Introduced by George Sayers.)

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The rate of secretion of 17-hydroxycorticosterone by the adrenal of the hypophysectomized dog prior to and following injection of

ACTH has been determined to elucidate the time-relationships concerned in the activation of adrenal secretion.

* This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service and by a research grant from the Upjohn Co.

[†] Experimental work was conducted in partial fulfillment of the Research Project Teaching Program at Western Reserve University School of Medicine.

Methods. Male dogs weighing 11.3 to 19.5 kg were anesthetized with sodium pentobarbital and hypophysectomized via the oral approach. The animals were maintained under light sodium pentobarbital anesthesia throughout the remainder of the experiment. At 3.25 to 12.7 hours after the hypophysectomy the left lumboadrenal vein was cannulated

for collection of adrenal venous blood. The adrenal vein and all collateral branches of the lumboadrenal vein were ligated. Seven to 10 ml of 1% heparin solution were injected intravenously. A small amount of heparin was added to each collection vessel. Control samples of adrenal venous blood were collected over 3 consecutive 10-minute intervals. ACTH[†] was then injected intravenously as follows: 1) 40 units were injected as the initial dose, 2) thereafter 8 units were injected every 10 minutes for 50 minutes. Adrenal venous blood was collected continuously during this period in samples representing 2-minute intervals for the first 10 minutes (with the exception of Exp. I in which the first post-ACTH sample was collected over a 10-minute interval) and 10-minute intervals thereafter. Each sample of adrenal venous blood was diluted with an equal volume of distilled water and extracted twice with a volume of chloroform equal to that of the blood-water mixture. Centrifugation was necessary to break the emulsion which formed during extraction. The residue from the chloroform extract was partitioned between 70% ethanol and hexane. Each 70% ethanol fraction was resolved by paper chromatography (48 hours) in a toluene-propylene glycol system(1). 17-Hydroxycorticosterone was located on the developed chromatograms with an ultraviolet lamp. The 17-hydroxycorticosterone was eluted with methanol and quantitatively analyzed by a modified Porter-Silber method(2). The results are expressed as μg 17-hydroxycorticosterone/kg body weight/minute. Recovery of 17-hydroxycorticosterone from chromatogram was determined and found to be consistently over 90%.

Results. The results of this study are summarized in Fig. 1. In 4 of the 5 experiments the post-hypophysectomy interval was greater than 4 hours and the control level of secretion of 17-hydroxycorticosterone was $.06 \pm .02 \mu\text{g}/\text{kg}/\text{minute}$. This rate is approximately 1/10 of the average ($.675 \pm .2 \mu\text{g}/\text{kg}/\text{minute}$) obtained from 4 intact anesthetized dogs bled under the same conditions(3). The low

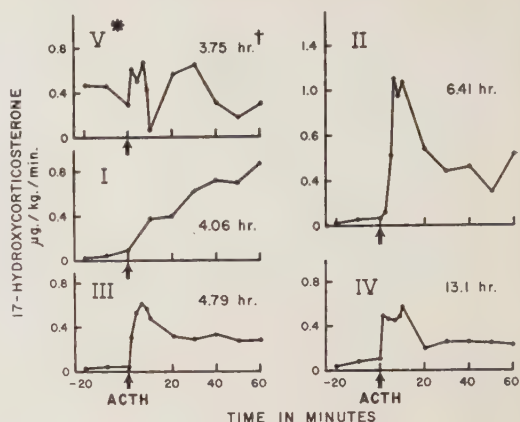


FIG. 1. Secretion of 17-hydroxycorticosterone by adrenal of hypophysectomized dog before and after intravenous ACTH as determined by analysis of adrenal venous blood.

* Exp. No. (see text).

† Time in hr between hypophysectomy and initial injection of ACTH.

level of secretion at 4 or more hours after hypophysectomy is in agreement with findings reported earlier from this laboratory(4). In Exp. V (3.75 hours post-hypophysectomy) the average control level of secretion was higher ($.416 \mu\text{g}/\text{kg}/\text{minute}$) than in the other experiments; the level declined in Exp. V during the 30-minute control period.

The rates of secretion of 17-hydroxycorticosterone 2 minutes after ACTH injection were significantly higher ($P < .05$) than the control rates. The over-all average for the 60 minutes following the initial injection of ACTH was $.502 \mu\text{g}$ 17-hydroxycorticosterone/kg/minute, which when compared with the average of $.675 \pm .2 \mu\text{g}/\text{kg}/\text{minute}$ found in intact anesthetized dogs(3) indicates that a near-normal level of secretion was obtained; however, the adrenal secretory response varied markedly among the experimental animals. In 3 experiments (II, III, IV, Fig. 1), the response was a burst of 17-hydroxycorticosterone secretion followed by a decline to an approximately constant level. In one experiment (V) the response was biphasic, consisting of a primary increase immediately following the first ACTH injection and a secondary increase occurring at 20 minutes. By contrast, in one experiment (I) the response consisted of a gradual increase in the rate of secretion

‡ Armour "Acthar", 1.4 units/ml in .9% NaCl made slightly acid with HCl.

over the 60-minute interval studied. The reasons for this variability in response are not clear at this time.

An important aspect of the problem of the response of the adrenal to ACTH is the relative role of increased *synthesis de novo* as compared to the *release of stored hormone*. The finding of a sudden increase of 17-hydroxycorticosterone secretion immediately after ACTH (Exp. II, III, IV, V) suggests the release of preformed 17-hydroxycorticosterone or the rapid conversion to this steroid of closely related precursors which may have accumulated in the gland during the period following hypophysectomy. Analysis of the steroid content of the gland during the post-hypophysectomy period may shed light on this important problem.

The data presented here permit an estimate of the speed with which the pituitary-adrenal system is capable of responding to a stressful stimulus. Increased pituitary ACTH secretion is known to occur within one minute after exposure of the animal to stress(5-7). The experiments reported here do not give an *exact* determination of the time-lag between the entrance of ACTH into the circulation and the beginning of increased secretion, but they indicate that it is less than 2 minutes. It seems evident that by 3 minutes (pituitary time-lag plus adrenal time-lag) after the application of a stressful stimulus the pituitary-adrenal system will have become activated to secrete increased amounts of corticosteroids. This finding is of interest in view of the work of Sayers and Sayers(8) who found a maximum decrease in the ascorbic acid content of the rat adrenal within 30 minutes after stress

or ACTH. Our findings are also in agreement with those reported by De Gurrpide(9) who found an increase in the 17-hydroxycorticosterone content of adrenal venous blood of the dog a few minutes after ACTH injection. They are also in agreement with the findings of Hechter *et al.*(10) who demonstrated an increased output of steroid by the isolated perfused bovine adrenal within 30 seconds after the addition of ACTH to the perfusate.

Summary. The rate of secretion of 17-hydroxycorticosterone by the adrenal of the dog as determined by chromatographic analysis of adrenal venous blood reached a low and apparently constant level within 4 hours after hypophysectomy. Two minutes after intravenous ACTH injection in these animals, the rate of secretion was significantly increased.

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Effect of Acetic Acid Hydrazide on Mammary Carcinoma 755 in C57 Black Mice.* (21234)

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Hydrazine derivatives are of theoretical interest in the chemotherapy of experimental tumors. As early as 1876 Fischer(1) noted the ease with which hydrazine compounds conjugate with carbonyl groups. These derivatives may conceivably upset biological oxidation systems important for the promotion of tumor growth, such as the citric acid cycle. Sixty-six hydrazine derivatives were previously reported by the authors as having no therapeutic effect on mouse leukemia(2). In the present experiments mammary carcinoma 755 was selected for study. This tumor is sensitive to the vit. B₆ antagonist, 8-azaguanine [5-amino-7-hydroxy-1H-v-triazolo(d)-pyrimidine](3). The inhibitory effect of acetic acid hydrazide [$\text{CH}_3\text{CONHNH}_2$] on this tumor is reported below.

Materials and methods. The drug was prepared in this laboratory. C-57 black mice averaging 20 g in weight were implanted subcutaneously with mammary adenocarcinoma 755. Therapy was started 24 hours later. Dosage was the maximum tolerated with slight weight loss. Acetic acid hydrazide was dissolved in water and thoroughly mixed with ground Purina Laboratory Chow, to make a final concentration of 0.15%. Each experiment of 7 to 20 mice was controlled by an equal number of animals which were given the same diet without the drug. After 11-14 days the surface area of the tumors was calculated from measurements of two diameters. The tumors and organs were examined for gross pathological and histological changes. All animals were weighed at the start and end of the experiment. As a positive control, 8-

TABLE I. Inhibition of Growth of Mouse Tumor 755 by Acetic Acid Hydrazide.

Compound	Surface area tumor relative to controls, %	Ratio wt gain, treat/controls	Ratio living, treat/controls
Acetic hydrazide	28	-1.7/+1.0	7/ 7
	27	-2.7/+2.2	7/ 7
	18	-2.0/+2.2	9/ 9
	15	-2.5/+1.3	19/20
8-Azaguanine	8	-.9/+3.8	7/ 8
	17	-1.7/+ .9	6/ 7

azaguanine,‡ 40 mg/kg was given daily intraperitoneally.

Results. The results are summarized in Table I. The surface area of the treated tumors was approximately 20% of the untreated control tumors. Inhibition produced by 8-azaguanine was more pronounced. No permanent effect was obtained; a week after the end of therapy the tumors had resumed growth. The mice showed approximately 10% weight loss; however only one animal died out of 45 treated. It is doubtful that this moderate amount of debilitation was the main cause of the tumor inhibition. The spleens and thymus glands were small compared to the tumor controls. The average weight of the spleen of the treated mice was 77 mg and the thymus 58 mg, which were 57% and 64% respectively, of the same organs in the untreated controls. Antopol, Glaubach, and Graff(4) have recently observed the spleens of mice inoculated with tumor 755 to be enlarged. Acetic hydrazide in the present experiments prevented this splenomegaly. Histological examination showed atrophic changes in the spleen and thymus. Otherwise no abnormal pathological or histological changes were observed in the tumors or organs of the treated animals. The absence of histological

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‡ The 8-azaguanine was provided through the courtesy of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

changes in the treated tumors suggests that the mode of action of acetic hydrazide on tumor inhibition is not a direct toxic action on the tumor cells. Three mechanisms of action have been considered. The hydrazide portion of the molecule may combine with a carbonyl group and remove an essential compound from the citric acid cycle, or the acetyl moiety can manifest itself through interference with active acetate formation. Busch(5) in experiments on the metabolism of acetate-1-C¹⁴ in tissues of tumor-bearing rats showed that utilization of the acetate molecule in the tumors was markedly diminished. Acetic hydrazide may further depress this utilization of acetate. In addition acetic hydrazide

might, conceivably, interfere with the activity of some amidases.

Summary. Acetic acid hydrazide caused moderate growth inhibition of transplanted mammary carcinoma 755. Possible modes of action are discussed.

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Fluorescent Antibody Studies with Agents of Varicella and Herpes Zoster Propagated *in vitro*.^{*} (21235)

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Investigation of varicella and herpes zoster viruses in the laboratory has been hampered by the lack of a susceptible experimental animal. An alternative approach was indicated by the observation(1) that specific cytopathic changes followed inoculation of roller tube cultures of human tissues with vesicle fluid materials from cases of varicella or herpes zoster. Serial propagation *in vitro* of agents apparently derived from these cases was accomplished, as demonstrated by the regular appearance in subcultures of cytopathic changes that were focal in nature and associated with the presence of intranuclear inclusion bodies. A peculiarity of the agents in

the culture system employed was the apparent failure of infectious material to appear in the fluid phase; therefore, tissue suspensions from infected cultures were utilized as inocula for passage *in vitro*. Initially this peculiarity posed technical problems in obtaining immunologic evidence of their identity. The fluorescent antibody technic developed by Coons and coworkers(2,3) was then applied in an effort to clarify the immunologic relationship of the agents under investigation.

This technic was first applied to problems dealing with the growth of viruses *in vitro* by Watson(4) in a study of mumps virus. She employed fluorescein-conjugated antimumps serum, and in general heretofore conjugates have been prepared with each type of antibody under investigation. Recently, one of us (AHC) prepared a fluorescein conjugate with antihuman gamma globulin rabbit serum for the purpose of detecting any specific antibody of human origin after combination with

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[†]This work was done during the tenure of a Career Investigatorship of the American Heart Association.

its homologous antigen.[‡] In the present study this antiglobulin conjugate was employed in an effort to detect virus in infected culture materials. Specifically the reaction between agents considered to be those of varicella or herpes zoster and homologous human antibody was investigated.

Materials and methods. Tissue culture materials infected with agents isolated from cases of varicella or herpes zoster were exposed to acute or convalescent phase sera collected from cases of varicella or herpes zoster, or to sera from cases of recurrent herpes simplex. To control the specificity of the reaction uninoculated tissues were included in each experiment, and, on occasion, tissues infected *in vitro* with herpes simplex virus were also studied. Cultures were prepared and maintained as previously outlined(1) in medium containing no human serum. Tissue fragments were planted in a plasma layer on long coverslips (No. 1; 11 x 50 mm) that were introduced into roller tubes. After cellular growth was well established the virus inoculum was added; the period of cultivation prior to inoculation varied from 6 to 15 days for cultures of human embryonic skin-muscle tissue and from 11 to 27 days for human foreskin tissue. The inoculum, in the case of the varicella and zoster agents, consisted of a tissue suspension prepared by grinding the cells removed from infected tissue cultures in nutrient medium: 0.1 to 0.3 ml was introduced per culture. That containing herpes simplex virus consisted of a 1:20 dilution in isotonic phosphate buffer (pH 7.1) of infected tissue culture fluid, and 0.1 ml was introduced per roller tube. All inoculated coverslip cultures were maintained until definite focal lesions appeared. In the varicella and zoster experiments the cultures were terminated as follows when cytopathic lesions had become apparent: one group on the 7th day after inoculation, 6 groups on the 8th day, 3 on the 10th, 2 on the 11th and one set each on the 13th, 15th and 19th days. Focal lesions appeared within 48 hours in the

preparations inoculated with herpes simplex virus and the cultures were harvested at that time. The coverslip preparations were removed and washed by dipping 3-4 times in isotonic phosphate buffer, and air dried in an incubator at 37°C for one to two hours. They were then covered with acetone in a Petri dish for 10 minutes and again dried. The relatively dense central area of each implanted tissue fragment was dissected free with the point of a scalpel and discarded, leaving the thinner outgrowth on the slip. The preparations were placed in Petri dishes at -15°C until exposed to serum and examined. The storage period usually was 1 to 5 days, although fluorescence was observed with preparations kept at -15°C for 8 days. Unsatisfactory results were obtained in one experiment with coverslip cultures stored for 50 to 60 days after drying. In each experiment a number of coverslips from uninoculated cultures were similarly handled. The procedure of examination for antigen in the coverslip cultures was as follows. Paired preparations infected with one strain of virus were overlaid for 30 minutes with a 1:10 dilution of human serum in phosphate buffer; one coverslip receiving acute phase and the other convalescent phase serum from the same patient. (In the early experiments, the human sera were twice absorbed with mouse liver powder as previously described(2), but it was ascertained that this step could be eliminated without resultant "nonspecific staining.") The coverslips were washed by gentle agitation in buffered saline (0.15 M NaCl containing 0.01 M phosphate, pH 7) for 10 minutes and then overlaid for 30 minutes with anti-human gamma globulin prepared in rabbits and labeled with fluorescein; this had been twice absorbed with dried mouse liver powder. The preparations were again washed in saline for 10 minutes and mounted by placing the inverted preparation on a drop of reagent glycerol containing 1 part in 10 of buffered saline on a slide. The stained cultures were examined under the fluorescence microscope and photographs taken as described(2,3,5).

Virus strains and sera studied. A. *Agents from cases of varicella.* Four strains were studied; 3 have previously been mentioned

[‡] Concurrently and independently a similar technic has been developed by Dr. David Gitlin at the Children's Medical Center, Boston, employing conjugated antigamma globulin sera.

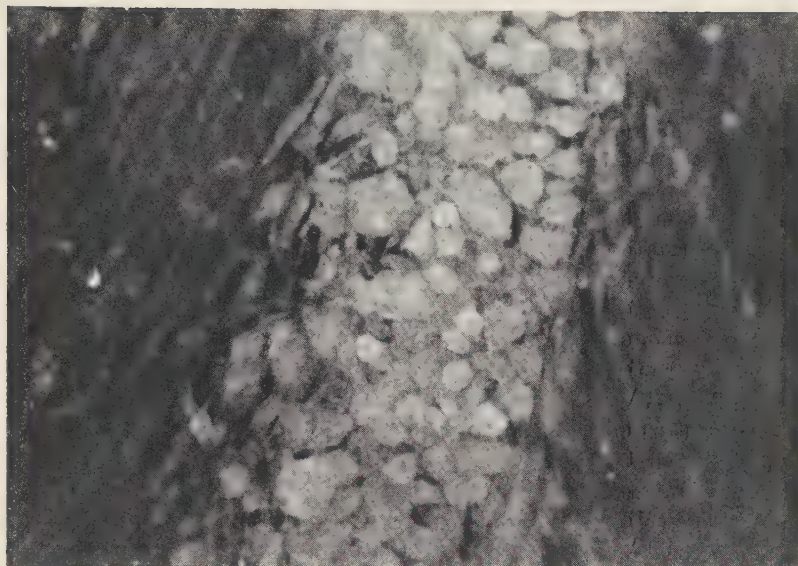


FIG. 1. Focal varicella lesion stained by fluorescent technic in human embryonic skin-muscle tissue culture; 10 days after inoculation with 14th passage Wel. strain material. Exposed to convalescent varicella serum #1555B. 250 \times .

(1). Inocula derived from the 10th to 12th tissue culture passage of the McE. strain were employed in four experiments; from the 11th to 14th *in vitro* passage of the Wel. strain in 2 experiments; and from the 3rd to 7th passage of the Pat. strain in 3 experiments. An additional strain, designated Cic., was isolated postmortem from the lung of a case of generalized varicella(6); in a single experiment with this strain, second culture passage material provided the inoculum. B. *Agents from cases of herpes zoster*. Three agents were employed. One strain, Sto., previously mentioned(1), was used as 5th culture passage material. Strain Blu., isolated *in vitro* from the vesicular eruption of a 37-year-old male with characteristic unilateral lumbar lesions, provided the inocula for 3 experiments in the form of 3rd to 9th culture passage material. Strain Bag., isolated from a 61-year-old woman with a typical thoracic lesion, was employed in a single experiment as 4th culture passage material. C. *Herpes simplex virus*. The Rod. strain, originally isolated by Dr. F. C. Robbins from the lung of a patient with fatal generalized herpes, was used as pooled infective fluids derived from the 4th tissue culture passage. D. *Sera studied*. Serum

specimens collected from 10 patients with varicella, from 5 with herpes zoster, and from 5 with recurrent herpes simplex were examined; they had been stored in the frozen state. Those from cases of herpes simplex were obtained through the courtesy of Dr. M. M. Stark and had been shown by him to possess high levels of herpetic complement fixing antibody(7). Dr. J. J. Finn, Jr. kindly provided the herpes zoster material.

Experimental. Infected coverslip preparations in the absence of serum as seen under the fluorescence microscope showed a faint blue-gray background luminescence that permitted visualization of the normal tissue and of the focal lesions, but showed no fluorescence. A total of 33 uninfected culture preparations examined in the presence of convalescent phase sera from cases of varicella or herpes zoster were similarly negative. Preparations infected with the agent of varicella when examined in the presence of sera collected before the 5th day of illness revealed minimal or no fluorescence, whereas in the presence of sera collected during early convalescence the focal collections of rounded cells were strongly fluorescent. As a further check on the specificity of the reaction, a preparation showing un-

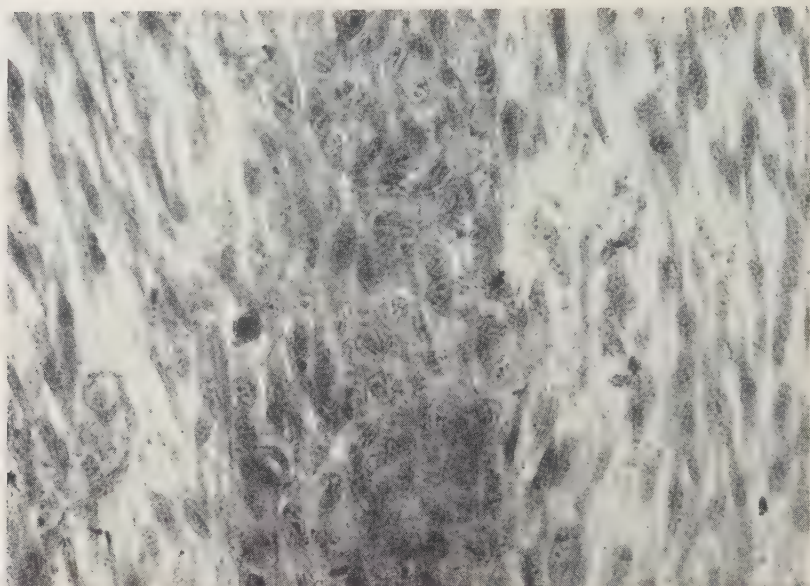


FIG. 2. Focal varicella lesion stained with H and E in culture of human embryonic skin-muscle tissue; 7 days after inoculation with 10th passage Wel. strain material. Fixed: Bouin's. 250 \times .

stained foci in the presence of acute phase sera was unmounted, washed, exposed to convalescent phase serum and conjugate, and re-examined; the foci then stained brilliantly. The pattern of fluorescence observed in cultures infected with the varicella and zoster strains was identical, being sharply limited to the focal collections of rounded cells. Fig. 1 pictures such an area stained by the fluorescence method after exposure to convalescent serum; for comparison, a comparable lesion stained with hematoxylin and eosin is shown in Fig. 2. Staining of cells infected with the agent of varicella as observed under higher magnification is pictured in Fig. 3 and 4. Interpretation on a cytologic basis of localization of the staining was difficult. In certain of the marginal fibroblasts adjacent to a cytopathic focus the nuclei were outlined with stained granules and there was a lesser degree of cytoplasmic staining. The periphery of those cells that had rounded up was usually brightly fluorescent, and such cells frequently contained irregular granular masses of fluorescent material; it was not clear whether these were cytoplasmic or nuclear in location, and while certain of the masses resembled stained

intranuclear inclusions a definite interpretation was not possible.

The fluorescent foci observed in preparations infected with herpes simplex virus in the presence of sera from cases of recurrent herpes were similar. However, the stained areas lacked the sharp demarcation observed with the other agents, and scattered stained cells were general throughout the preparation. There appeared to be less rounding of infected cells, and in those wherein morphology was little altered, definite cytoplasmic staining was observed with a more intense nuclear staining.

The degree of fluorescence observed with the varicella, zoster and simplex agents in the presence of acute and convalescent phase homologous and heterologous human sera was compared. The reactions were graded from 0 to 4+, with \pm indicating minimal and 4+ indicating intense fluorescence, as summarized in Table I. Results similar to those listed, but not recorded, were obtained with varicella strain Cic. when tested with paired varicella and paired zoster sera and with an additional zoster strain, Bag., when tested with paired zoster sera. The several varicella and zoster strains reacted in an identical manner with

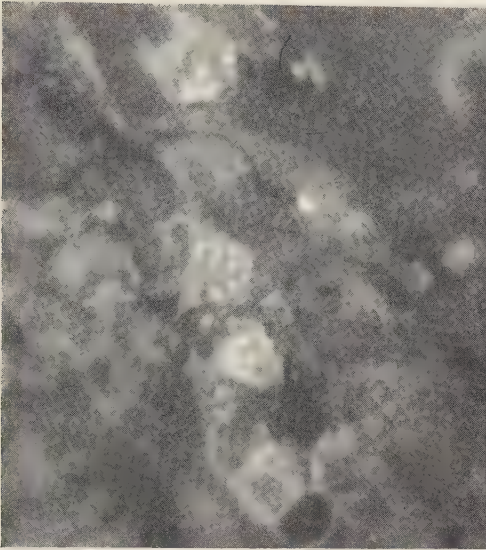


Fig. 3

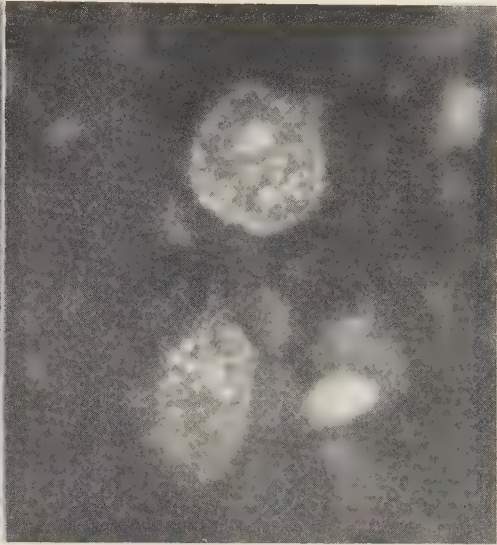


Fig. 4

FIG. 3. Edge of focal varicella lesion stained by fluorescent method in culture of human foreskin tissue; 14th day after inoculation with McE. strain. Exposed to convalescent varicella serum #1006C. About 320 \times .

FIG. 4. Preparation as in Fig. 3. High power view of cells in varicella lesion. 600 \times .

paired sera from either disease. Antibody combining with both agents was absent or present in low titer in acute phase sera from varicella or zoster cases and then increased during the course of the illness. On the other hand, no comparable rise in antibody was observed when acute and convalescent phase sera from cases of varicella or zoster were compared in the presence of simplex virus. In the paired sera from one zoster case, a high level of antibody to simplex virus was observed in both specimens. The sera from recurrent cases of herpes simplex that were examined all reacted strongly in the presence of the homologous virus.

Discussion. Previously, on the basis of morphologic evidence, serial propagation *in vitro* of agents apparently derived from cases of varicella and of herpes zoster was described. Now, through the use of the fluorescent antibody technic, immunologic evidence has been adduced that provides additional support for the thesis that the agents are those responsible for varicella and herpes zoster. Also, the close relationship, if not identity, of agents isolated from the two clinical entities is further suggested by their similar behavior when em-

ployed as antigen in the fluorescent antibody method. Antibody capable of reacting with the varicella or zoster antigen appeared in the serum of patients with varicella around the 3rd to 5th day after the appearance of vesicles. It is of interest that Amies(8) observed the development of agglutinins for varicella elementary bodies after a similar interval. Recently corroborative immunologic evidence has been obtained in support of the findings here described. From tissue culture materials infected with the agent of varicella a satisfactory complement fixing antigen has been prepared(9); with this antigen, the development of complement fixing antibody has been demonstrated in paired serum specimens obtained from cases of varicella and from cases of herpes zoster.

The observations here reported indicate the potential usefulness of an alternative technic for the demonstration of virus propagated *in vitro*. In preliminary experiments we have observed that cultured human myometrial cells infected with Type I poliomyelitis virus fluoresce following exposure to convalescent phase sera and then to the antiglobulin conjugate(10). The method may prove par-

TABLE I. Results of Fluorescent Antibody Staining of Tissue Cultures Infected with Varicella, Herpes Zoster and Herpes Simplex.

Day ill- ness*	Agents used to infect cultures—					
	Varicella strains			Zoster strains		Simplex strains
	McE.	Wel.	Pat.	Blu.	Sto.	
Sera from varicella cases:						
1	0	0	0	0		
23	4+	3+	+	3+		
3	0		0			
39	+		+			
1	0	0	0	0	0	
9	3+	4+	3+	3+	4+	
3			±	+		
13			4+	4+		
3	0	0	±	+	0	±
9	4+	4+	4+	4+	4+	+
2	0	0				
650	0	0				
5	+	±	+	+	±	
52	4+	3+	3+	4+	4+	
1	±	0	0	0	0	+
7	4+	4+	4+	4+	3+	+
2			0			
9			3+			
35			2+			
5			2+			
9			3+			
31			3+			
Sera from zoster cases:						
2			0	+		
41			3+	3+		
2	0			0	0	±
51	3+			2+	+	±
4	±			0	0	4+
27	3+			4+	4+	4+
8	4+			4+	3+	
32	4+			4+	4+	
6				+		
28				3+		
Sera from simplex cases (recurrent):						
			±			4+
			±			4+
	0			0	0	2+
	±			0	0	3+
				0		3+
				0		3+
				+		3+
				+		3+
				±		4+
				±		4+

* Dated from appearance of vesicles.

ticularly useful for the detection of viruses that do not manifest overt cytopathogenicity *in vitro*.

Summary. Tissue culture preparations infected with agents originally derived from the eruptive lesions of cases of varicella and herpes zoster, as well as control preparations infected with the virus of herpes simplex, were studied by a modification of the fluorescent antibody technic. Employing the infected preparations as antigen, fixation of antibody from human sera derived from cases of varicella, herpes zoster or herpes simplex was detected by the use of a fluorescent antihuman gamma globulin conjugate. Antibody reacting with the varicella and herpes zoster antigens to an almost identical degree appeared during convalescence in serum specimens derived either from cases of varicella or from cases of herpes zoster. Antibody reacting with herpes simplex virus was demonstrated uniformly only in a group of sera derived from cases of recurrent herpes simplex. Immunologic evidence was thus obtained to support the thesis that the etiologic agents of varicella and herpes zoster have been isolated and propagated *in vitro*.

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Effect of Lecithin, Cholesterol and Cardiolipin on Mumps Virus Hemolysin.* (21236)

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The hemolysin of mumps virus(1) has been shown to exhibit many of the characteristics ascribed to enzymes(2,3) and to be distinct from the mumps hemagglutinin(2-5). In the course of experiments studying the nature of the mumps hemolysin, it was found to possess certain properties in common with lecithinase A, such as similar pH and temperature optima, inhibition by urethane and by high concentrations of calcium and magnesium, and the failure of potassium cyanide and sodium fluoride to affect its hemolytic action. However, the mumps viral hemolysin differs from lecithinase A in that it is heat labile while lecithinase A is heat stable(5). Because of this similarity of the mumps viral hemolysin to a lecithinase, it was of interest to test the action of lecithin on the viral hemolysin. Cholesterol and cardiolipin were studied also to determine if they would affect the hemolytic action of mumps virus.

Materials and Methods. A strain of egg-adapted mumps virus was used(1-5). Embryonated hens' eggs, which had been incubated previously for 8 days at 37.5°C, were inoculated amniotically with 0.1 ml of a 10⁻² dilution of the seed virus in broth. After inoculation, the eggs were incubated 5-6 days at 35°C, chilled in the refrigerator and the infected fluids harvested. These virus-infected fluids were placed in screw-cap test tubes and stored at -40°C until used. Before use these fluids were rapidly thawed and dialyzed for 24 hours at 4°C against phosphate buffered saline (pH 7.0-7.2). This buffered saline was used as the diluent for all reagents and tests in this study. Tests for hemolytic and hemagglutinating activities of the virus were carried out with the methods previously described (2,3), using a 0.5% suspension of chicken

erythrocytes for the hemagglutination tests and a 2% suspension for the hemolysin tests. In previous experiments(2,3), percentage hemolysis readings of less than 10% were found to be unreliable so these values are disregarded and the hemolytic titer is taken as the highest dilution of virus producing at least 10% hemolysis. Furthermore, one-tube differences in the hemolysis or hemagglutination tests are within experimental error and therefore not significant. Stock solutions of lecithin, cholesterol and cardiolipin were prepared in absolute alcohol and were diluted for use in buffered saline with agitation to secure even dispersion of small size particles. Lecithin was prepared from egg yolk according to Pangborn's method(6,7). Cholesterol was purchased from Eastman Kodak Co., and the cardiolipin was generously supplied by Dr. M. C. Pangborn(8,9). These materials suspended in buffered saline were used as diluents for the virus and for the cell controls in the hemagglutination and hemolysis tests to determine their possible inhibitory effects. Similar dilutions of alcohol were used in all experiments to observe any possible action of alcohol on erythrocytes or virus or their interaction to affect either hemagglutination or hemolysis. In studies in which the test compounds were allowed to interact with the chicken erythrocytes before the virus was added, 20 ml volumes of washed erythrocytes were added to an equal volume of a buffered saline suspension of the test compound and mixed thoroughly. One aliquot was placed in the refrigerator at 4°C and the other at 37°C for 3 hours. At the end of this incubation period, the erythrocytes were washed 3 times and a 0.5% and a 2% suspension prepared which was added in equal volumes to each of two serial dilutions of virus for tests respectively of its agglutinative and its hemolytic activities. In certain experiments, virus was allowed to react with cells before the addition

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TABLE I. Effects of Lecithin, Cholesterol, and Cardiolipin on Hemagglutination and Hemolysis by Mumps Virus. Eleven experiments.

Inhibitor, mg/ml	Hemolytic test							Hemagglutination test	
	Dilutions of virus fluid						Cell control	Titer	Cell control
	8	16	32	64	128	256			
Lecithin									
.0	72*	53	34	23	12	0	0	2048†	—
.3	73	52	33	21	13	0	0	2048	—
.0	43	33	23	18	11	10	0	1024	—
.4	25	16	9	0	0	0	0	1024	—
.0	55	41	28	21	16	13	0	1024	—
.9	30	20	9	6	0	0	0	1024	—
.0	55	43	29	18	11	0	0	512	—
5.0	21	12	0	0	0	0	0	256	—
Cholesterol									
.0	42	37	26	18	13	0	0	1024	—
.05	34	22	14	10	0	0	0	>2048	±
.0	55	41	28	21	16	13	0	1024	—
.09	44	28	19	13	0	0	0	>2048	+
.0	55	43	29	18	11	0	0	4096	—
.5	23	0	0	0	0	0	0	>8192	+
Cardiolipin									
.0	58	42	28	18	12	0	0	2048	—
.04	53	33	23	15	11	0	0	>2048	+
.0	24	17	11	0	0	0	0	256	—
.05	14	0	0	0	0	0	0	>2048	+
.0	28	21	15	10	0	0	0	512	—
.1	16	11	0	0	0	0	0	>2048	+

* % of hemolysis determined colorimetrically.

† Reciprocal of highest dilution of virus producing hemagglutination.

—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

of the various compounds by making serial dilutions of virus in a 2% suspension of erythrocytes and placing these suspensions in the refrigerator for 3 hours. At the end of this period, the cells were washed 3 times with cold buffered saline and resuspended in buffered saline to make a 0.5% and a 2% suspension of virus-adsorbed red cells to which an equal volume of fluid containing the test compound could be added and its effect on hemagglutination and hemolysis, respectively, observed.

Results. 1. *Effect of lecithin, cholesterol and cardiolipin on mumps virus hemagglutination and hemolysis.* Table I presents data showing that lecithin, cholesterol and cardiolipin inhibit the hemolytic action of mumps virus. Controls on these tests showed that the effect was not due to the alcohol used to dissolve these compounds, nor was their action the result of a change in pH, which is known to affect the mumps hemolysin(2,3), since pH

measurements of the fluids revealed no significant alterations. The lowest concentration of lecithin exhibiting at least a four-fold decrease in hemolysin titer contained 0.4 mg/ml (0.00514 M) and increasing the concentration up to 5 mg/ml greatly enhanced this inhibitory action. None of the quantities of lecithin tested produced any significant effect on the hemagglutinating activity of the virus. Cholesterol caused a 4-fold reduction of viral hemolysis at a concentration of 0.09 mg/ml (0.000232 M), and smaller quantities had no significant effect. At this level (0.09 mg/ml) cholesterol produced agglutination of the erythrocytes *per se*. Cardiolipin in the concentrations of 0.04 mg/ml to 0.1 mg/ml caused spontaneous agglutination of the red cells, though it inhibited the viral hemolysin when levels of 0.05 mg/ml were reached, but concentrations of cardiolipin exceeding 0.1 mg/ml were hemolytic *per se*.

In other experiments it was shown that the

TABLE II. Results of Reaction of Inhibitor and Virus before Addition of Erythrocytes and Hemolysis.

Inhibitor	mg/ml	Hr at 4°C	Hemolytic test									Hemagglutination test	
			Dilutions of virus fluid								Cell control	Titer	Cell control
			2	4	8	16	32	64	128	256			
0	0	0	68*	52	43	33	23	18	11	10	0	1024†	—
	0	18	65	53	40	29	20	14	10	10	0	"	—
Lecithin	.4	0	55	29	25	16	11	10	0	0	0	"	—
		18	52	26	10	0	0	0	0	0	0	"	—
	.7	0	48	31	18	10	0	0	0	0	0	"	—
		18	33	10	0	0	0	0	0	0	0	"	—
Cholesterol	.05	0	67	54	41	29	20	14	10	0	0	>2048	±
		18	63	48	32	17	10	0	0	0	0	"	±
	.09	0	48	48	36	26	17	10	0	0	0	"	+
		18	46	46	27	13	0	0	0	0	0	"	+

* % of hemolysis determined colorimetrically.

† Reciprocal of highest dilution of virus producing hemagglutination.

—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

inhibitory activity of lecithin, cholesterol and cardiolipin at their minimal effective concentrations was markedly enhanced when they were present simultaneously. This additive effect enhanced their suppressive action on viral hemolysis at least 16 times.

2. *Action of inhibitor on virus before addition of cells.* In their suppressive action on viral hemolysis these compounds might exert an effect primarily on the virus, the erythrocyte, or on their interaction to produce lysis of the erythrocyte. Experiments were therefore undertaken to determine if their addition to the virus prior to its admixture with the erythrocytes would enhance the inhibitory action. A typical experiment in which lecithin and cholesterol were allowed to interact with serial dilutions of virus overnight at 4°C is presented in Table II.

Exposure of virus to the inhibitors for 18 hours at 4°C enhanced their suppressive action on the viral hemolysin, though control tests indicated that the viral hemolysin did not deteriorate under these conditions. Lecithin at 0.4 mg/ml exhibited the most marked increase of inhibitory activity for the viral hemolysin, though the viral hemagglutinin was unimpaired, indicating that the virus still combined as readily with erythrocytes. The action of cholesterol on the viral hemolysin also increased under these conditions, but any possible action on the viral hemagglutinin was

obscured by its own agglutinative property.

3. *Action of inhibitors when added to erythrocytes before exposure to virus.* Since the compounds under study might produce their inhibitory actions on viral hemolysis by altering the surface of the erythrocytes, the effect of treating the red cells with inhibitors on their subsequent hemolysis by the virus was studied by the method described and results, using lecithin, are summarized in Table III. Pretreatment of erythrocytes with lecithin (0.4 mg/ml) at 37°C caused a 4-fold decrease in viral hemolysin titer, but interaction of lecithin and red cells at 4°C produced no effect. Neither type of treatment had any effect on the viral hemagglutinin, thus indicating that the virus could adsorb to the erythrocytes in an unimpaired manner. Furthermore, since hemagglutination tests on the supernatant fluids in the hemolysin tests failed to show any impairment of release of virus from the lecithin-treated cells, the elution process was not interfered with. Lecithin apparently interacts with erythrocytes at 37°C but not at 4°C in a manner which interferes with their hemolysis by mumps virus, but it does not affect the capacity of the virus to adsorb to and elute from these erythrocytes. Since the erythrocytes were thoroughly washed after contact with the lecithin before use, it may be suggested that they were "coated" with lecithin.

TABLE III. Influence of Pretreatment of Erythrocytes with Lecithin upon Hemolytic Activity of Virus.

Treatment of erythrocyte for 3 hr mg/ml	Temp., °C	Hemolytic test								Hemagglutination test	
		Dilutions of virus fluid							Cell control	Titer	Cell control
.0	37	84*	70	58	40	25	16	10	0	2048†	—
		64‡	16	16	4	2	—	—	—	—	—
.4	"	67	52	37	23	14	0	0	0	"	—
		128‡	32	16	4	—	—	—	—	—	—
.0	4	90	81	64	43	30	21	15	0	"	—
		64‡	16	8	4	2	—	—	—	—	—
.4	"	86*	75	56	39	24	14	10	0	"	—
		128‡	64	8	4	2	—	—	—	—	—

* % of hemolysis determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.
‡ Reciprocal of hemagglutination titer of supernatant fluid after hemolysis test.
—, No hemagglutination.

Similar treatment of erythrocytes with cholesterol (0.14 mg/ml) and cardiolipin (0.04 mg/ml) at 4°C or 37°C had no effect on the hemolytic activity of mumps virus.

Since it was thought that lecithin might be present on the erythrocyte surface as a "coating" after washing of the erythrocytes, it was of interest to see if there was any specificity of inhibition of viral hemolysis associated with the nature of a substance coating the surface of an erythrocyte. A purified polysaccharide† prepared from *Escherichia coli*, which was shown to coat erythrocytes by rendering them agglutinable by a homologous *E. coli* immune serum, was allowed to react with chicken erythrocytes in the same manner as lecithin. These coated erythrocytes were hemolyzed and agglutinated by mumps virus in an unimpaired manner though their surfaces were coated by the polysaccharide, since they were readily agglutinated by the *E. coli* antiserum.

4. *Action of inhibitors after virus was adsorbed to erythrocytes.* In the experiments described to this point, the inhibitors were always present at the time of adsorption of virus to the erythrocyte, and it is of importance to determine whether or not they can interfere with the viral hemolytic reaction after this combination has occurred. Virus was allowed to interact with erythrocytes, as described herein, at 4°C which permits adsorption to red cells but no hemolysis(2,3).

† Obtained through the courtesy of Mr. B. B. Wiley.

These erythrocytes were then washed to remove any virus not attached to the red cells and the inhibitors added in appropriate concentrations with the results presented in Table IV.

With addition after adsorption of virus to the erythrocyte, none of the inhibitors had any effect on viral hemolysis in concentrations studied, though they were present in the suspending fluid during the period of incubation at 37°C when hemolysis occurred.

Discussion. Since cholesterol and lecithin are believed to be components of the outermost surface of the red cell wall, their inhibitory effects on hemolysis by mumps virus are of special interest. In concentrations of 0.4 mg/ml of lecithin and 0.09 mg/ml of cholesterol these compounds reduced the hemolytic activity of mumps virus. The specificity of this inhibitory action is subject to question, however, since cardiolipin, a phospholipid isolated from heart tissues, also has been shown to inhibit the mumps hemolysin.

If, as earlier studies suggest, this viral hemolysin is an enzyme(1-5), it may be that in attacking the cell wall of the erythrocyte it has some special affinity for the constituent cholesterol or lecithin. These substances, if present in the reactive hemolytic system, possibly combine with the viral hemolysin first and thus render it unable to affect the erythrocyte surface. Since hemagglutination takes place in an unimpaired manner in the presence of lecithin, it is again suggested, in line

TABLE IV. Effect of PreadSORption of Virus to Erythrocytes upon Inhibitory Capacity of Compounds.

Erythrocytes	Inhibitor	mg/ml	Hemolysis test								Hemagglutination test	
			Dilutions of virus fluid							Cell control	Titer	Cell control
			2	4	8	16	32	64	128			
Virus preadsorbed	0	.0	52*	44	40	37	20	16	10	0	512†	—
<i>Idem</i>	Lecithin	.4	50	39	33	22	16	12	0	0	256	—
	Cholesterol	.07	52	43	35	24	19	13	10	0	>512	±
	Cardiolipin	.05	56	46	36	26	19	11	0	0	>512	+

* % of hemolysis determined colorimetrically.

† Reciprocal of highest dilution of virus producing hemagglutination.

—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

with previous experimental evidence(2,3) that hemolysis occurs subsequent to hemagglutination during elution of virus from the cell. When the inhibitors are allowed to come in contact with the virus before addition of the erythrocytes, their suppressive reaction is enhanced and most markedly with lecithin, which suggests that the viral hemolysin does react with lecithin and cholesterol. Furthermore, lecithin appears to act by binding the virus particle in some way to prevent hemolysis without affecting its adsorption to the red cell surface, resulting in hemagglutination.

If lecithin is an important reactive constituent of the erythrocyte surface for viral hemolysis, "coating" of the cell with excess lecithin should reduce hemolysis. Experiments carried out in this study showed that pretreatment of erythrocytes with lecithin at 37°C rendered them less susceptible to hemolysis by mumps virus, but similar exposure at 4°C was without effect, as was also similar treatment at 37°C with cholesterol or cardiolipin. If lecithin does "coat" the erythrocytes, the temperature at which exposure occurs appears important. Absence of any effect on the susceptibility of the erythrocytes treated with lecithin to hemagglutination by the virus indicated that virus could still combine with the erythrocytes. In addition, these experiments demonstrated that virus also eluted in an unimpaired manner from these treated red cells. Furthermore, a polysaccharide prepared from *E. coli*, which could be shown to "coat" the erythrocyte, had no effect on viral hemolysis, indicating some specificity for the action

of lecithin.

The role of the inhibitors was further clarified by the experiments in which virus was allowed to combine with erythrocytes before the inhibitors were added and the temperature raised to levels permitting the hemolytic action to occur. All 3 substances had no significant effect under these conditions. This suggests that the virus had already combined with the critical elements in the erythrocyte surface and thus hemolysis could occur as the virus eluted from the erythrocyte(2,3), in spite of the presence of the inhibitory compounds.

In all of these experiments the selective inhibitory effect of lecithin on the viral hemolysin as contrasted to the viral hemagglutinin supports the concept that these are separate and distinct properties of mumps virus.

Summary. 1. Lecithin, cholesterol, and cardiolipin reduce the activity of mumps virus hemolysin when present prior to but not after the combination of virus and erythrocyte and their inhibitory effects at minimal concentrations were shown to be additive. Lecithin does not affect the hemagglutinating activity of the virus, but the effects of cholesterol and cardiolipin cannot be ascertained as they agglutinate erythrocytes *per se*. Pretreatment of erythrocytes with lecithin but not with cholesterol or cardiolipin at 37°C rendered them less susceptible to lysis by mumps virus. 2. These results present additional evidence for the separate identity of the viral hemolysin and the viral hemagglutinin and suggest that, in the erythrocyte cell wall, lecithin and

cholesterol may be important constituents in its reaction with the viral hemolysin.

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Cholesterol Balance Studies in Mice with Modified Thyroid Activities.* (21237)

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It was observed recently in this laboratory (1), that in cholesterol-fed rats, thyroid administration significantly reduces plasma and liver cholesterol levels, in agreement with earlier findings(2-4). These effects cannot be attributed to a decrease in the rate of endogenous cholesterol formation, as thyroid hormone stimulates synthesis of the sterol(5-7). Therefore, the possibility was considered that mechanisms leading to an increased rate of sterol disposal are enhanced in the hyperthyroid state. Our work was undertaken to investigate, whether, in the mouse, the thyroid hormone accelerates the catabolism of cholesterol.

Methods. The total cholesterol balance was determined essentially as described recently (8). In the first experiment, female mice, 5-6 weeks old, were used.[‡] All animals were fed purina chow supplemented with 1% sulfasuxidine and 0.05% streptomycin§ for one day prior to the experimental run, to prevent or

reduce cholesterol destruction by the intestinal flora(8). During the entire balance period of 16 days, all mice of both experimental groups were kept individually in metabolism cages, and fed a diet containing the components mentioned above, 0.65% cholesterol, and other supplements as indicated.|| In addition, the animals of the hyperthyroid group were given, at onset, one intraperitoneal injection of 50 μ g of thyroxine, and then, fed 0.4% desiccated thyroid USP with their diet during entire balance period. In a second experiment, male mice (Swiss Colony Strain)¶ 5-6 weeks old were used. Prior to the experimental run, all animals were fed Purina Chow supplemented with 0.5% thiouracil** and the mentioned

§ Sulfasuxidine—Sharp and Dohme, Division of Merck and Co., Streptomycin hydrochloride—E. R. Squibb and Sons.

|| Experimental diet: Purina chow (ground), 81.35%; yeast (strain G, Anheuser-Busch), 10%; dextrin, 2%; Wesson oil, 4.5%; cholesterol, .65%; desiccated ox bile (USP), .25%; sulfasuxidine, 1%; streptomycin, .25%. Cholesterol was dissolved in Wesson oil before being mixed with the diet.

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† Predoctoral fellow of Life Insurance Medical Research Fund.

‡ Mice were obtained from California Caviary, Los Angeles.

TABLE I. Effects of Thyroid and Thiouracil on the Total Cholesterol Balance of Mice under Different Dietary Conditions.

Exp.	Feeding conditions	Cholesterol content of diet, %	Endocrine supplement	No. of mice	Total cholesterol balance			
					Avg input, mg	Avg recovery* mg	%	P for difference
1	Restricted	.65	Thyroid	8	313	250	80 ± 5	<.02
			None	6	320	302	94 ± 1	
2	"	"	Thyroid + thiouracil	10	290	268	92 ± 3	<.001
			Thiouracil	7	269	297	110 ± 1	
3	<i>Ad libitum</i>	.15	Thyroid + thiouracil	8	92	115	125 ± 4	<.001
			Thiouracil	7	83	85	103 ± 3	

* The stand. errors of the means were calculated using the formula: $S_M = \sqrt{\frac{\Sigma(x^2)}{n(n-1)}}$.

x = deviation from the mean; n = No. of animals. The P values were calculated using Fisher's Table of t.

antibacterial drugs for 12 days, and then divided into 2 groups. One group continued on the same regimen, while the other was fed 0.4% thyroid powder (USP) with the thiouracil containing diet, for a further pretreatment period of 3 days. During the balance period of 10 days, the mice were fed diets described for the second pretreatment period, further supplemented with cholesterol and the other supplements mentioned.¹¹ In both experiments, all animals received the same amount of diet twice daily. In a third experiment, animals used, and endocrine treatments were the same as in Exp. II. The dietary regimen, however, was different, *i.e.*, a diet containing only 0.15% cholesterol was fed *ad libitum* for 4 days. During that time the food intake was determined. In all 3 experiments, excreta were collected during the balance period, and the mice were then sacrificed. The carcasses and excreta were hydrolysed in 30% (w/v) KOH solution in 50% (v/v) alcohol, and then extracted with diethyl ether. The solid residues remaining after hydrolysis and extraction of feces were re-extracted for 10 hours in Soxhlet apparatus, first with 95% (v/v) alcohol and then with ether. The total cholesterol content of extracts was then determined using a modified Schoenheimer-Sperry procedure⁽⁹⁾. The values obtained are usually considered to represent essentially cholesterol, although a contribution of other unsaturated sterols cannot be completely excluded⁽¹⁰⁾. In some in-

stances, the livers were excised at autopsy and their cholesterol content was determined separately. The values for the overall cholesterol balance were calculated from the total cholesterol input (carcass at onset + food consumed) and the total cholesterol recovery (carcass at end + excreta). The carcass cholesterol contents at onset were computed, using body weights of experimental animals at beginning of balance period, and the average carcass cholesterol concentrations of additional animals ("onset controls") treated in identical manner and sacrificed at same time. Four to 6 mice were used per group as onset controls; individual variations within groups were insignificant.

Results. The thyroid treatment produced symptoms typical of hyperthyroidism. *E. g.*, the physical activity, nervousness, water consumption and mortality, and, in Exp. 3, the food intake and fecal excretion were significantly increased.

The results obtained for the total cholesterol balance are shown in Table I. In Exp. 1 and 2, hyperthyroid mice were compared with normal and hypothyroid animals, when pair-fed restricted amounts of a diet containing 0.65% cholesterol. In the groups supplemented with thyroid, the cholesterol output was significantly smaller than the sterol input, and also significantly smaller than the recovery in the respective controls. In Exp. 2, the overall cholesterol recovery of the animals fed thiouracil exceeded 100%. The liver

TABLE II. Effects of Thyroid and Thiouracil on Cholesterol Content of Carcass and Excreta of Mice under Different Dietary Conditions.

Exp.	Feeding conditions	Cholesterol content of diet, %	Endocrine supplement	Avg total cholesterol content			
				Of carcass*		Of food eaten per 24 hr, mg	Of excreta per 24 hr,† mg/g body wt
				At on-set,† mg	At end,† mg		
1	Restricted	.65	Thyroid	38 ± 2	32 ± 1	17.2	1.29 ± .09
	"	"	None	36 ± 2	37 ± 2	17.8	1.44 ± .03
2	"	"	Thyroid + thiouracil	85 ± 4	74 ± 4	20.5	.85 ± .02
	"	"	Thiouracil	62 ± 3	88 ± 2	20.7	1.03 ± .01
3	<i>Ad libitum</i>	.15	Thyroid + thiouracil	62 ± 3	64 ± 2	7.5	.53 ± .02
	"	"	Thiouracil	66 ± 4	59 ± 2	4.4	.29 ± .01

* In Exp. 2, mortality rate was relatively high in the group supplemented with thyroid; somewhat heavier mice, obtained as replacements for the animals lost in this way, survived better; as a consequence, the avg body wt was somewhat greater for the hyperthyroid group.

† For calculation of the stand. errors see Table I.

cholesterol values are not shown separately as their contribution to the values for the overall balance was of minor extent.

In Exp. 3, the cholesterol balance of hyperthyroid mice was compared with that of thiouracil-treated animals under a different dietary regimen, *i.e.*, when fed *ad libitum* a diet relatively low in cholesterol. The results indicate that alteration of the nutritional state of the mice profoundly modified the action of the thyroid hormone on the cholesterol balance. In the hyperthyroid group, the cholesterol recovery was significantly larger than the sterol input, and larger than that for hypothyroid animals, in contrast to the data recorded for Exp. 1 and 2, where reverse relationships were observed.

When the effects of thyroid on the cholesterol contents of carcass and excreta are examined separately, a similar trend is observed (Table II). Under conditions of a restricted feeding of a high cholesterol diet, the hyperthyroid groups showed a decrease in rate of cholesterol excretion, as compared to normal (Exp. 1) or hypothyroid (Exp. 2) mice. The carcass cholesterol content was only slightly decreased by thyroid administration; in the hypothyroid animals, however, this value was found significantly increased under these dietary conditions (Exp. 2). Apparently, the thyroid treatment prevented such an accumulation of tissue cholesterol, in spite of a relatively high cholesterol intake.

When a diet relatively low in cholesterol was fed *ad libitum*, the hyperthyroid animals ex-

creted cholesterol at a markedly increased rate, as compared to the mice treated with thiouracil alone (Exp. 3). The carcass cholesterol levels, however, were not significantly modified under these conditions.

Discussion. The results of the first 2 experiments were interpreted to indicate that the thyroid hormone can stimulate the degradation or modification of cholesterol. This effect was observed under conditions of a restricted intake of a diet relatively high in cholesterol. The authors are aware of only one report dealing with similar observations, an abstract of a paper by Hurxthal and Perkin(11). However, no experimental data were given in this paper and the dietary conditions employed were different. On the basis of turnover studies Rosenman *et al.* also concluded that the thyroid hormone enhances cholesterol destruction(12).

Investigators have shown that administration of thyroid hormone causes a reduction of tissue cholesterol concentrations elevated as a consequence of cholesterol feeding(1-4). The above mentioned results suggest that the mechanisms responsible for this phenomenon include reactions leading to the destruction or chemical modification of cholesterol, perhaps in addition to those stimulating the excretion of the sterol(12).

The results of the third experiment, on the other hand, were interpreted to indicate that, under conditions of *ad libitum* feeding of a diet relatively low in cholesterol, the thyroid hormone stimulated primarily endogenous

cholesterol synthesis and cholesterol excretion, in agreement with earlier observations(5-7, 12).

It is obvious, therefore, that the thyroid hormone may influence different phases of cholesterol metabolism and that the experimental conditions, in particular, the dietary regimen determine which effect predominates in a particular case.

Under the conditions of the first 2 experiments, the total caloric intake was inadequate for the requirement of a hyperthyroid animal; therefore, a condition of relative starvation or semi-starvation existed. During starvation, the rate of endogenous cholesterol synthesis is markedly reduced, as shown by Hutchens, *et al.*(13), Tomkins and Chaikoff(14) and Frederickson, *et al.*(15). Furthermore, the cholesterol intake was relatively high, and this also tended to reduce endogenous cholesterol synthesis, in line with reports by Gould and Taylor(16), Tomkins and co-workers(17) and Frantz, *et al.*(18). Thus 2 factors operated which are known to depress mechanisms responsible for cholesterol synthesis, and any effect which thyroid hormone would otherwise have exerted in this direction, was apparently inhibited by these circumstances. Therefore, the experimental conditions were favorable for a demonstration of another action of this hormone, *i.e.*, its influence on cholesterol destruction or conversion, and this effect predominated.

In the third experiment, on the other hand, the total caloric intake was more nearly adequate in the hyperthyroid group (*ad libitum* feeding), and the cholesterol intake was relatively low. Therefore, an entirely different metabolic pattern was observed, *i.e.*, the major action of the hormone was directed towards stimulating endogenous cholesterol synthesis and cholesterol excretion, and these effects predominated, obscuring any influence the hormone might have had on cholesterol destruction.

Summary. The total cholesterol balance was determined in mice fed 0.4% thyroid, in normal controls, and in mice fed 0.5% thiouracil. When a diet containing 0.65% chole-

sterol was offered in restricted amounts (paired feeding), the thyroid hormone stimulated destruction or chemical conversion of cholesterol. However, when a diet low in cholesterol was fed *ad libitum*, the thyroid hormone stimulated primarily the endogenous synthesis and the excretion of the sterol. The relation of the nutritional state of the animal to the mode of action of the thyroid hormone on the metabolism of cholesterol is discussed.

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Diminished Plasma 17-Ketosteroid Concentration in Pregnancy.* (21238)

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Measurements of urinary 17-ketosteroid excretion in healthy pregnant women have indicated that there is little or no increase in the excretion of these metabolites during human gestation(1). Women with hypoadrenocorticism, who have essentially no urinary 17-ketosteroids prior to pregnancy, show a progressive increase in the urinary excretion of these steroids. This increase is especially marked in the last 3 months of pregnancy. By term their urine excretion values are equivalent to what is found in the non-pregnant female without hypoadrenocorticism, and after delivery urinary 17-ketosteroid excretion again falls off to negligible amounts(2-4). Thus by difference it might be postulated that there is a lessened production of 17-ketosteroids by the mother's own adrenal cortex during the course of normal pregnancy. To throw light on this possibility measurements have been made of plasma neutral 17-ketosteroid concentrations in pregnant women at term.

Methods. Blood samples were obtained from normal, pregnant females at term who had just been admitted to the delivery floor of the Syracuse Memorial Hospital. In nearly all cases the patients delivered within 24 hours of admission. Control specimens were obtained from normal, adult members of the medical, technical and nursing staff of the hospital. Blood samples were drawn without respect to time of day. Oxalate was used as an anticoagulant, and the plasma was separated by centrifugation, after which samples were stored at 0° F. until analyzed. Plasma neutral 17-ketosteroids were determined by a method which has been reported elsewhere(5). The results were expressed as μg of 17-ketosteroids per 100 ml of plasma, dehydroepiandrosterone acetate being used as a standard.

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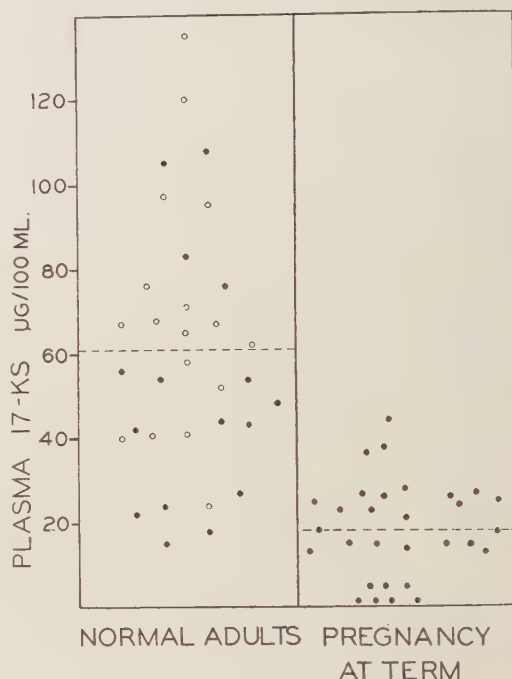


FIG. 1. Plasma 17-ketosteroid values on normal adults compared with pregnant women at term. Open circles = men. Closed circles = women. Dotted lines = mean values.

Results. Fig. 1 shows the plasma 17-ketosteroid concentration data obtained on the control and experimental groups. The control group, consisting of 17 men and 16 women showed a mean value of $61 \mu\text{g}$ per 100 ml (S. E.[†] = 5). Due to the spread of values in the population sample tested, there was found to be no significant difference between the data on men and non-pregnant women ($P > .05$).

Data on 30 pregnant women at term showed a mean value of $18 \mu\text{g}$ 17-ketosteroids per 100 ml plasma (S.E. = 2). This mean was significantly lower than the mean value of the controls ($P < .001$).

Discussion. The finding of diminished

[†] S.E. = Standard Error of the Mean.

plasma 17-ketosteroid concentration in late pregnancy, together with urine excretion data previously referred to(1-4), suggest that there is a slackening off during pregnancy of 17-ketosteroid production by the maternal adrenal cortex. Recent studies of concentration gradient of 17-ketosteroids between cord and maternal plasmas have shown there were consistently higher values in cord plasma than in plasma samples taken at same time from the mother(6). We have also become aware of a simultaneous study by Migeon, who found that cord plasma shows the same dehydroepiandrosterone and androsterone values as the normal adult, whereas dehydroepiandrosterone concentration in maternal plasma is lower than in non-pregnant adults(7). Our findings lead to supposition that inner cortex of fetal adrenal and/or the placenta produce the 17-ketosteroids of late pregnancy. Whether this is "compensatory" as result of cessation of maternal 17-ketosteroid production, or whether maternal reduction is secondary to events occurring within the fetal circulation is an interesting, but as yet unanswered question. Renal clearance studies of 17-ketosteroids in normal pregnancy are needed to clarify the relationships among following data: (a) 17-ketosteroids in fetal (cord) plasma at adult levels; (b) lowered 17-ketosteroid levels in maternal plasma; and (c) slight or no increase of urinary 17-ketosteroid excretion during pregnancy.

Summary. 1. Plasma neutral 17-ketosteroid determinations were made on control group of 33 normal men and women, and on experimental group of 30 pregnant women at

term. 2. Adult controls showed mean plasma value of 61 $\mu\text{g}/100\text{ ml}$ (S.E. = 5). No significant difference was found between values obtained on normal men vs. normal, non-pregnant women ($P > .05$). 3. Pregnant women showed a mean plasma value of 18 $\mu\text{g}/100\text{ ml}$ (S.E. = 2). This value is significantly lower than the mean of control adults ($P < .001$). 4. From the results of plasma analyses it is concluded that there is a physiological reduction in *maternal* elaboration of 17-ketosteroids during the last 24 hours of pregnancy. The data of other workers on urinary excretion of 17-ketosteroids in pregnancy(1-4) suggest that this finding may hold for the last third of pregnancy. Renal clearance studies will be necessary before final conclusions can be drawn. 5. It is postulated that the inner cortex of the fetal adrenal and/or the placenta take over production of 17-ketosteroids during the latter part of pregnancy.

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Influence of Age upon Response to Meningococcal Endotoxin in Rabbits. (21239)

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In previously reported studies on the mechanism of the generalized Schwartzman reaction in this laboratory (1-6), young albino rabbits, weighing between 1 and 1.5 kilos each, were routinely employed in the majority of experiments. Recently, in the course of an investigation involving larger rabbits, a paradoxical difference was noted in the vulnerability of young and mature animals to the lethal effect of a single injection of meningococcal endotoxin. Doses of endotoxin which produced little or no evidence of systemic intoxication in the young rabbits were observed to be lethal within a few hours after injection in older animals of the same stock. In view of the increased susceptibility of older rabbits to a single injection, a comparison was made of the reaction to 2 doses of endotoxin, spaced 24 hours apart. It was found that the incidence of the generalized Schwartzman reaction, as indicated by the incidence of bilateral cortical necrosis of the kidneys, was much lower in the older group of animals.

Materials and Methods. Hybrid albino rabbits of both sexes were obtained from a single breeder, housed in air-conditioned and humidity-controlled quarters, and maintained on a diet of Purina rabbit pellets and water. Rabbits of 2 age ranges were used: the group designated "large" in the text to follow were between 10 and 12 weeks of age, and weighed 2.0 to 3.0 kilos, and the "small" animals were 4 to 7 weeks old and weighed 0.5 to 1.0 kilo.

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TABLE I. Influence of Age on Mortality in Rabbits following a Single Intravenous Injection of Meningococcal Endotoxin.

Avg age (wk)	Avg wt (k)	Mortality					
		Dilution of endotoxin*					Totals
		1-20	1-40	1-80	1-160	1-320	
11	2.42	5/8†	5/8	3/8	4/8	3/8	20/40
4.5	.62	0/8	1/8	0/8	0/8	0/8	1/40

* 2 cc of indicated dilution of endotoxin inj. intravenously.

† Numerator—No. dead within 18 hr. Denominator—No. in group.

The endotoxin was derived from a strain of meningococcus (44B) obtained from Dr. Gregory Schwartzman, Mount Sinai Hospital, New York. The method of preparing the material has been described (1). Various dilutions of endotoxin were made in sterile physiological saline, and injected intravenously in a volume of 2.0 cc; no adjustment of dosage for the body weight was made. Colloidal iron in the form of saccharate of iron oxide ("Proferin", supplied by Sharpe and Dohme, Inc.) was employed in some of the experiments in order to enhance the lethal effect of endotoxin, as described in a previous report (5). The material was injected intravenously in a dosage of 2.0 cc per kilo, 2 hours before the injection of endotoxin.

Results. Lethal Effect of Single Injection of Endotoxin. A titration of meningococcal endotoxin was performed in 40 large and 40 small rabbits, employing 5 different dilutions of endotoxin. A summary of the results is shown in Table I. Within 2 to 3 hours after the injection, all of the large animals became obviously ill, with weakness, labored respirations, and varying degrees of prostration, and within 18 hours 20 of the 40 were dead. In contrast, although some of the small animals were relatively inactive for several hours after the injection, few showed evidences of severe intoxication and only one died. When the dose of endotoxin is estimated in terms of

body weight, the data indicate that the small rabbits tolerated at least 50 times the amount of endotoxin which was lethal for the large animals. The majority of deaths in the large rabbits occurred within 12 hours after injection, and after this many of the surviving animals began to recover from the shock-like condition produced by the endotoxin. By the end of 24 hours the survivors appeared to be completely well, and no additional deaths occurred after this time. It is evident that a factor of individual susceptibility, in addition to the age of the rabbits, was of importance in determining the outcome. It will be noted in Table I that although each of the doses of endotoxin was lethal for large rabbits, there was no dose which caused death in all animals. Moreover, the dosage could not be correlated with the proportion of animals killed; 2-fold or greater increases in endotoxin did not cause a proportional increase in mortality.

Lethal effects of endotoxin following an injection of colloidal iron saccharate. Reticulo-endothelial "blockading" agents such as thorotrast, trypan blue(2), colloidal carbon, and colloidal iron saccharate(5), were previously demonstrated to enhance to a remarkable degree the lethal effect of endotoxin, an effect which was interpreted to indicate that the reticuloendothelial system may be involved in natural protection against endotoxin. It seemed important to determine whether the lethal effect of endotoxin was similarly influenced by these materials, in rabbits of different ages. Groups of large and small rabbits were given an injection of colloidal saccharate of iron oxide in a dose of 2 cc/kilo. Two hours later the animals were injected with 2 cc of different dilutions of meningococcal

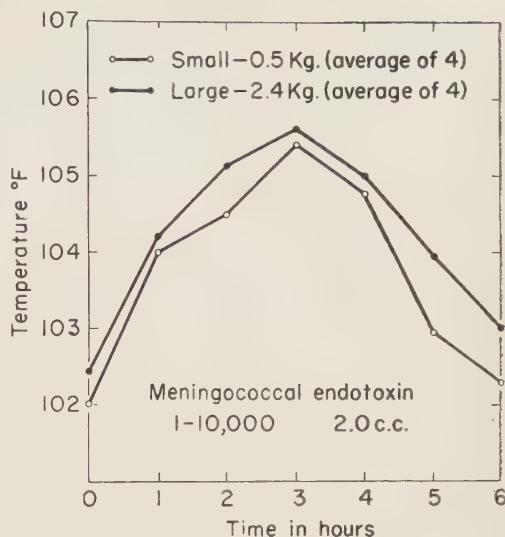


FIG. 1. Temperature response of large and small rabbits given 2 cc, 1-10000 meningococcal endotoxin intravenously. Each point represents avg in four rabbits.

endotoxin. The results are shown in Table II. It will be seen that death was produced in both groups by small doses of endotoxin. The vulnerability of the small animals was now equal to, or slightly greater than that of the larger group, indicating that enhancing effect of colloidal iron is greater in the young rabbits.

Influence of age on pyrogenic effect of endotoxin. An intravenous injection of gram-negative bacterial endotoxin produces in rabbits an abrupt temperature elevation, rising to a maximum in one to 3 hours, and subsiding over a 6- to 8-hour period. Groups of small and large rabbits were given intravenous injections of various amounts of meningococcal endotoxin, and the rectal temperature was determined hourly for the ensuing 6-hour period. The result of an illustrative experiment, in which 4 large and small rabbits received 2 cc of 1-10000 dilution of endotoxin, are shown in Fig. 1. Similar results were obtained with other dilutions of endotoxin. In contrast with the striking difference in the lethal effect of endotoxin for large and small rabbits, no significant differences in the febrile response could be demonstrated.

Influence of age on incidence of generalized Schwartzman reaction. The generalized Schwartzman reaction, characterized by bi-

TABLE II. Influence of Age on Mortality in Rabbits Injected with Endotoxin following an Injection of Colloidal Iron Saccharate.

Avg age (wk)	Avg wt (k)	Mortality				
		Dilution of endotoxin*				Total
		1-320	1-640	1-1280	1-2560	
11	2.60	3/4†	2/4	2/4	0/4	7/16
5	.59	3/4	3/4	4/4	1/4	11/16

* Rabbits were given colloidal iron saccharate intravenously, 2 cc/kilo, and 6 hr later, 2 cc of the dilution of endotoxin indicated.

† Numerator—No. dead within 18 hr. Denominator—No. in group.

TABLE III. Influence of Age on the Incidence of the Generalized Shwartzman Reaction.

Batch of endotoxin*	Size of rabbits†	No. in group	—No. of deaths‡—		No. with renal cortical necrosis
			After 1st inj.	After 2nd inj.	
A	Large	39	20	11	3 (7.7%)
	Small	38	1	2	26 (68.4%)
B	Large	29	8	7	0 —
	Small	28	1	2	8 (28.5%)

* See text.

† Large rabbits 11 wk old, average 2.5 kilo. Small rabbits were 5 wk old, average 0.6 kilo.

‡ Two intravenous injections of endotoxin were given, 24 hr apart. No. of rabbits dying within 18 hr after each inj. are recorded.

lateral cortical necrosis of the kidneys, is produced in rabbits by the administration of 2 intravenous injections of endotoxin spaced 18-24 hours apart. In previous studies of the reaction conducted in this laboratory, young rabbits have been used in most experiments. In view of the increased vulnerability to a single injection of endotoxin encountered in older rabbits, it was of interest to determine whether susceptibility to the generalized Shwartzman reaction was comparably influenced by age. Groups of large and small rabbits were given 2 intravenous injections of endotoxin, spaced 24 hours apart. Two batches of endotoxin, differing in potency, were employed. Batch A was known to produce bilateral renal cortical necrosis in 70% of small rabbits; the incidence with batch B was approximately 30%. All surviving rabbits were sacrificed 24 hours after the second injection, and the incidence of the generalized Shwartzman reaction was determined by gross examination of the kidneys. The results, summarized in Table III, indicate that the incidence of the generalized Shwartzman reaction is much lower in large rabbits, despite the increased vulnerability to the lethal effect of endotoxin in these animals. The low incidence with batch A was partly attributable to the fact that the majority of large rabbits died within a few hours after the first or second dose of endotoxin, before sufficient time had elapsed for the development of renal cortical necrosis. However, the results with batch B, which permitted survival in half of the group but caused renal necrosis in none, suggest an actual decrease in susceptibility to the reaction in older rabbits.

Discussion. It has been demonstrated that immature rabbits, weighing less than one kilo,

are much less vulnerable to the primary lethal effect of meningococcal endotoxin than larger, more mature animals. Doses greater than 50 times the amount which killed large rabbits were tolerated with little or no evidence of illness by the small animals. The difference in susceptibility was eliminated by the intravenous administration of colloidal iron prior to endotoxin, which caused a greater degree of enhancement of the lethal effect in the small rabbits.

The influence of age on the response of animals to various effects of bacterial products has been the subject of several studies. Zahl, Hutner, and Cooper (7), studying the effects of *Shigella paradysenteriae* endotoxin in mice, showed that when the lethal dose of endotoxin was calculated on the basis of body weight, the young mice appeared to be more resistant than the older ones. However, there was no significant difference in the absolute dose of endotoxin causing death, disregarding body weight, in the 2 groups.

The effects of various products of gram-positive organisms have also been investigated in animals of different ages. Burky (8) found that young rabbits were more resistant to the lethal effect of staphylococcal filtrates than older ones. Parish and Okell (9) injected hemolytic streptococcal filtrate ("scarlet fever toxin") intravenously into rabbits of age groups comparable to those used in the present experiments. The young animals tolerated amounts of the material which were lethal to the older ones.

The difference between the response of large and small rabbits would appear to be based upon one of 2 factors. Either the small animals are in possession of a mechanism for defense which becomes less effective during

maturation, or the young are lacking in a vulnerable or "sensitive" mechanism which appears during maturation. The observation concerning the effect of colloidal iron on the reaction to endotoxin is compatible with the first interpretation. It has been suggested on the basis of other studies(2,6,12) that the reticuloendothelial system is concerned in the protection of normal animals against endotoxin. An intravenous injection of materials known to be taken up by the reticuloendothelial cells, such as thorotrast, trypan blue, colloidal carbon, or colloidal iron, creates temporarily a condition of markedly enhanced vulnerability to the lethal effect of very small doses of endotoxin. If the special resistance of young rabbits were the result of a relatively greater activity of the reticuloendothelial system in these animals, it might be expected that interference with the functioning of this system would eliminate the resistance. It was found that an injection of colloidal iron prior to toxin enhanced the vulnerability of both large and small rabbits, and at the same time eliminated the difference between the 2 groups by a relatively greater effect on the small animals. It seems possible that the resistance of younger rabbits may be related to a function of the reticuloendothelial system, but in the absence of more definitive information concerning the actual effect of colloidal iron on this system, the matter must remain speculative.

That there may be dissociation between the various physiological effects of endotoxin has been suggested by other studies(10). The results of the present study tend to support the idea that one response to endotoxin may be independent of another. Despite the considerable differences in the lethal effect in the 2 age groups, no demonstrable differences in the febrile reaction to small amounts of endotoxin was encountered.

As to the generalized Schwartzman reaction, produced by 2 successive intravenous doses of endotoxin, the results obtained also indicate that more than one mechanism is involved in the effects of endotoxin. The small rabbits, relatively invulnerable to the primary lethal effect of a single injection of endotoxin, showed a high incidence of renal cortical

necrosis after 2 injections, while the older animals, which were much more easily killed by one dose, were relatively insusceptible to the reaction.

For practical purposes, the low incidence of the generalized Schwartzman reaction in mature rabbits indicates that such animals are not suitable experimental subjects for investigation of this reaction.

Summary. A comparison was made of the response to meningococcal endotoxin in young (4-7 weeks) and more mature (10-12 weeks) rabbits, with the following results: 1. Young rabbits were relatively unaffected by a single intravenous injection of endotoxin, in doses more than 50 times the amount which caused prostration and death in older animals. 2. The febrile reaction to small doses of endotoxin was not significantly different in the 2 groups of rabbits. 3. An intravenous injection of colloidal iron saccharate enhanced the lethal effect of endotoxin in all rabbits, and eliminated the difference in susceptibility between young and older animals. 4. The generalized Schwartzman reaction, with bilateral cortical necrosis of the kidneys, occurred in a high proportion of young rabbits after 2 intravenous injections of endotoxin. This reaction could not be elicited in more than 10% of the older rabbits.

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Effect of Cortisone on Response to Endotoxin in Mature Rabbits. (21240)

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In previous studies in this laboratory of the effects of cortisone and other agents(1-6) on the response to endotoxin, small rabbits, 4-7 weeks in age and weighing between one and 1.5 kilo, were used in most experiments. In animals of this size, cortisone produced an effect which was analogous to one stage of the generalized Schwartzman reaction: a single intravenous injection of endotoxin, in cortisone-treated rabbits, resulted in bilateral cortical necrosis of the kidneys. The observations were interpreted to indicate an interference by cortisone with a protective mechanism, perhaps involving the reticuloendothelial system, against the vascular necrotizing action of endotoxin(6).

In view of the difference in the response to endotoxin in large and small rabbits demonstrated in the previous paper(7), an investigation of the effect of cortisone in large animals was undertaken. It was found that pretreatment with this material caused 2 separate effects. One was similar to that reported earlier with small rabbits: a single injection of endotoxin was followed by the development of renal cortical necrosis, but with an incidence lower than previously observed in small animals. The second effect was to prevent completely the reaction of early prostration and death produced by endotoxin in the older rabbits. By adjusting the doses of cortisone and endotoxin it was possible to dissociate the 2 effects, so that protection against the early lethal response without the subsequent de-

velopment of renal necrosis could be demonstrated.

Material and methods. The rabbits were of the same stock as the "large" animals employed in experiments described in the preceding paper(7). Meningococcal endotoxin was prepared by a method described elsewhere(1). Cortisone was obtained from Merck and Co., in a preparation for intramuscular injection containing 25 mg/cc.

Experimental. Effect of cortisone on early lethal effect of endotoxin. A group of 24 large rabbits, weighing 2.5 to 3.0 kilos each, received cortisone each day for 3 days in an intramuscular injection of 10 mg per kilo. Twenty-four animals of the same size were used as untreated controls. On the third day, meningococcal endotoxin was injected intravenously in a dose known to be sufficient to cause prostration and death in the majority of large rabbits. The outcome is shown in Table I. The general appearance of the cortisone-treated animals was strikingly different from the untreated controls. They remained active and alert, continued to feed, and were not distinguishable from normal animals. In contrast, all of the untreated rabbits exhibited obvious weakness and lethargy beginning about an hour after the injection, and developed varying degrees of prostration, with diarrhea, labored respirations and inability to remain upright. Within less than 18 hours after the injection of endotoxin 18 of the untreated group were dead, as compared with 3 of the cortisone-treated animals.

Renal necrosis in cortisone-treated rabbits. On the day following endotoxin injection, the situation appeared to be reversed. The surviving rabbits of the untreated group, which had been severely prostrated during the early hours of the experiment, seemed to be entirely recovered. On the other hand, some of the cortisone-treated animals had become apathetic and disinclined to feed. At the end of

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TABLE I. Effect of Pretreatment with Cortisone on Response to Meningococcal Endotoxin in Large Rabbits.

Group	No. of rabbits†	Death‡	Renal cortical necrosis
Cortisone-treated*	24	3	9
Untreated	24	18	0

* Cortisone 10 mg/kilo daily for 3 days prior to an intravenous inj. of meningococcal endotoxin.

† All rabbits were between 10 and 12 wk old, and weighed 2.5-3 kilos.

‡ Refers to No. dying within 24 hr after endotoxin. All animals were sacrificed at 24 hr.

24 hours after endotoxin, all were sacrificed and the kidneys examined. The results were included in Table I. Of the 24 treated animals, 9 had bilateral cortical necrosis of the kidneys. This lesion was not present in any of the untreated group of rabbits.

Protection by cortisone against death, without renal necrosis. In previous studies with small rabbits it had been learned that a pretreatment period of 3 days with cortisone was required for the consistent production of renal necrosis by a single dose of endotoxin(6). In order to determine whether a smaller dose of cortisone could protect against the early lethal effect of endotoxin without the subsequent occurrence of renal lesions, the following experiment was performed. Two groups of 6 large rabbits each were given an injection of 2 cc of a 1-20 dilution of meningococcal endotoxin. Six hours prior to this injection, one group received an intramuscular injection of 10 mg cortisone. The results are summarized in Table II. All of the untreated rabbits became prostrated within 1 to 2 hours, and 3 were dead within less than 12 hours. All of the cortisone-treated rabbits remained free of symptoms during the entire period of 24 hours after the injection, and when sacrificed at this time had no kidney lesions. The results indicate that a single dose of cortisone, 6 hours

TABLE II. Effect of a Single Injection of Cortisone 6 Hours before Endotoxin.

Group	No. of rabbits	Death	Renal necrosis
Cortisone-treated*	6	0	0
Untreated	6	3	0

* Cortisone, 10 mg, one inj. 6 hr prior to intravenous inj. of meningococcal endotoxin.

before endotoxin, is sufficient to provide protection against the early reaction of prostration and death. Renal cortical necrosis did not occur under these conditions. These findings suggest that the two effects of cortisone on the response to endotoxin may involve separate mechanisms. In other experiments, unsuccessful attempts were made to reverse the primary lethal reaction by injecting cortisone 1-2 hours after endotoxin, at a time when the rabbits were already beginning to show evidences of prostration. No effect on the symptoms or mortality could be demonstrated in this circumstance, with doses of cortisone as high as 25 mg per kilo.

Effect of cortisone on lethal reaction to colloidal iron followed by endotoxin. In previous experiments it was found that the early lethal effect of endotoxin was much enhanced when rabbits were given an intravenous injection of colloidal iron, thorotrast, trypan blue, or colloidal carbon a few hours prior to the injection of endotoxin(3-5). In addition, it was observed that a high proportion of animals receiving such combinations of colloidal materials and endotoxin developed renal cortical necrosis. The effect of pretreatment with cortisone was studied in the following manner: Two groups of 10 rabbits each were employed. In one group cortisone was given for 3 days, in a daily intramuscular injection of 10 mg per kilo. On the third day, both groups received colloidal iron saccharate ("Proferrin") in a dose of 2 cc per kilo, followed 2 hours later by 2 cc of a 1-80 dilution of meningococcal endotoxin. The results are indicated in Table III.

In the 10 control rabbits, not treated with cortisone, death occurred within a few hours in 7. One animal which survived until the next day had bilateral cortical necrosis of the kidneys. In contrast, only 1 cortisone-treated rabbit developed early prostration and died, while 7 developed renal necrosis during the following 24 hours.

The results suggested, as in the preceding experiments, that the two effects of cortisone on the response to endotoxin may be separate events. The enhanced early lethal reaction was inhibited, but the development of renal cortical necrosis was not prevented. In fact,

TABLE III. Effect of Cortisone on Lethal Effect of Endotoxin following Colloidal Iron Saccharate.

Group*	No. of rabbits	Death	Renal necrosis
Cortisone-treated	10	1	7
Untreated	10	7	1

* All rabbits received 2 cc colloidal iron saccharate ("Proferrin") intrav., 2 hr before meningococcal endotoxin. The cortisone-treated group received 10 mg cortisone/kilo daily for the 3 preceding days.

the incidence of renal necrosis was observed in other experiments with colloidal iron and endotoxin to be consistently higher in cortisone-treated animals than in untreated controls. This was due in part to the protection by cortisone against early death, with a consequently greater proportion of rabbits surviving for a long enough period to develop the renal lesion, and in part to the previously demonstrated capacity of cortisone itself to bring about renal necrosis after a single injection of endotoxin.

Discussion. Two separate and perhaps independent effects of cortisone on the response to endotoxin in large rabbits have been demonstrated in these experiments. The first of these is to protect against the early reaction of prostration, shock and death which is produced by endotoxin in mature rabbits. The second, described in detail earlier(6), is to bring about the lesions characteristic of the generalized Schwartzman reaction with a single injection of endotoxin.

When cortisone is administered in a sufficient amount, with a pretreatment period of 3 days before endotoxin, both actions are simultaneously demonstrable in the same experiment. The animals withstand the early effects of endotoxin, but develop renal necrosis during the next 24 hours. But when only one injection of cortisone is given, 6 hours before endotoxin, protection against early death occurs without the subsequent appearance of renal lesions.

Both properties of endotoxin—the production of prostration and early death, and the development of renal necrosis—are exhibited when the injection of endotoxin is preceded by certain colloidal materials such as saccharated iron oxide. The occurrence of shock and early

death is prevented by cortisone, but the subsequent development of renal cortical necrosis is not inhibited and is, in fact, to some extent augmented.

Studies by other workers have suggested that natural resistance to the lethal action of endotoxin in some way involves the adrenal gland. Lewis and Page(8) showed that adrenalectomized rats were extremely vulnerable to typhoid vaccine, and were protected by treatment with cortisone. In rabbits an antipyretic action of cortisone has been reported with several different endotoxins(9). Spink and Anderson(10) have shown that mice are protected against the lethal action of brucella endotoxin by cortisone; no renal lesions were encountered in this species.

On the other hand, the property of cortisone to bring about renal necrosis with a single injection of endotoxin cannot be interpreted to indicate that the adrenal cortex plays an essential role in the pathogenesis of the generalized Schwartzman reaction. In recent studies by Good and associates(11) it was found that this reaction could be produced by 2 injections of endotoxin in totally adrenalectomized rabbits, without supportive hormone therapy.

Summary. 1. Treatment with cortisone prevented the early reaction of prostration and death produced by an intravenous injection of endotoxin in mature rabbits. 2. In some of the rabbits pretreated for 3 days with large doses of cortisone, bilateral cortical necrosis of the kidneys occurred within 24 hours after endotoxin. However, when a single injection of cortisone was given 6 hours before endotoxin, protection against prostration and death was demonstrable without the subsequent development of renal necrosis. 3. The early lethal reaction caused by small amounts of endotoxin following an injection of colloidal iron saccharate was prevented by cortisone. Cortisone did not protect against the development of renal cortical necrosis in these animals.

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Cold-Precipitation by Heparin of a Protein in Rabbit and Human Plasma. (1241)

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The earliest pathological event in the generalized Schwartzman reaction, which is produced in rabbits by two intravenous injections of gram negative bacterial endotoxin, is the appearance of intravascular deposits of homogeneous, eosinophilic material with the staining properties of fibrinoid, within the lumen of glomerular capillaries and small vessels of the spleen, liver, and lungs, and in the walls of the coronary arteries(1,2). The morphological situation of the material indicates that it is derived from the circulating blood, and the observation that its deposition in the glomerular capillaries is prevented by heparin(3) suggests that the coagulation mechanism may be involved in its development. In the course of an investigation into possible precursors of fibrinoid, heparinized plasma was obtained from rabbits at various time intervals after an intravenous injection of endotoxin. It was noted that chilling of such plasma specimens

at 4°C was followed within less than an hour by the precipitation of gelatinous, flocculent material resembling an incomplete plasma clot. Warming the plasma to 37°C resulted in rapid solution of the material, and chilling again caused precipitation to occur. No cold-precipitation occurred in citrated plasma or in serum. Heparin-precipitable material was not demonstrable in the plasma of a majority of normal rabbits. However, it was encountered in the plasma of normal human beings and, in greater amounts, in the plasma of patients with acute rheumatic fever.

The present paper is a preliminary account of the conditions under which the heparin-precipitable material is demonstrable, with certain observations suggesting that it may be derived from fibrinogen.

Materials and methods. Young hybrid albino rabbits, weighing 1 to 1.5 kilos, were used in all experiments. The animals were maintained on Purina rabbit pellets and water. The following endotoxins were employed: meningococcal "agar washings" toxin prepared as previously described(1) from a strain of meningococcus (44B) supplied by Dr. Gregory Schwartzman, Mount Sinai Hospital, New York, a polysaccharide endotoxin from *S. marcescens*, supplied by Dr. Murray Shear, National Cancer Institute, Washington, and a purified *Sh. paradysenteriae* endotoxin supplied by Dr. Walter Goebel, Rockefeller Insti-

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TABLE I. Cold-Precipitation by Heparin of a Protein in Rabbit Plasma.

Endotoxin*	Time of bleeding	No. of rabbits	Cold-precipitation†	
			Heparin conc.—	
			1 mg/cc	.1 mg/cc
	Before endotoxin	20	1	3
Meningococcal	15 min. after endotoxin	10	0	1
	30 " " "	10	1	1
	1 hr " "	20	16	20
	2 " " "	20	20	20
	4 " " "	20	19	20
	6 " " "	10	8	10
	24 " " "	20	5	8
<i>S. marcescens</i>	2 " " "	6	6	6
<i>Sh. paradysenteriae</i>	2 " " "	6	6	6

* The following doses of endotoxin were employed: Meningococcal—2 cc of a 1-200 dilution; *S. marcescens*—0.2 mg; *Sh. paradysenteriae*—0.1 mg.

† Figures refer to No. of animals whose heparinized plasma showed a flocculant precipitate after 2 hr at 4°C.

tute, New York. Dilutions of each endotoxin were made in pyrogen-free saline. All injections were made intravenously, in doses described below. The rabbits were bled by cardiac puncture, using various concentrations of heparin or sodium citrate as anticoagulant. Sterile, pyrogen-free pyrex tubes were used for all manipulations of blood or plasma. Plasma was obtained from whole blood by centrifugation at 2800 rpm (1400 x g) for 15 minutes at room temperature, in a horizontal centrifuge. Heparin solutions containing 10 mg per cc were obtained from 3 commercial sources: Heparin Sodium (Upjohn), Liquaemin (Parke Davis) and Heparin Sodium (Vitarine Company). The preparations showed no differences in the property of causing cold-precipitation in plasma.

Heparin-Precipitable Protein in Rabbit Plasma. Blood was obtained from 20 normal rabbits, and from these and other groups of animals at various times after an intravenous injection of 2 cc of a 1-200 dilution of meningococcal endotoxin. At each bleeding, two 5 cc samples of blood were placed in tubes containing, respectively, 5.0 and 0.5 mg of heparin dissolved in 0.5 cc physiological saline. The blood was centrifuged immediately, at room temperature, and each plasma specimen was divided into 2 equal portions. One part was kept at 37°C, and the other placed in a 4°C refrigerator. The results are summarized in Table I. In all of the plasma samples obtained 2 or 3 hours after the injection of endo-

toxin, cold-precipitation occurred within less than an hour after chilling the tubes. No precipitate appeared in plasma kept at 37°C. In most instances, the plasma became turbid within 15-20 minutes after chilling, and during the next 15 minutes numerous small, glistening floccules appeared throughout the sample. These floccules then coalesced to form an opaque, gelatinous mass which settled to the bottom of the tube. The appearance of precipitates one hour after chilling is illustrated in Fig. 1. When tubes containing freshly precipitated material were warmed to 37°C, the precipitate went back into solution within a few minutes, and when chilled again, precipitation again occurred. In plasma chilled for 24 hours or longer, the precipitates were more solid in appearance and contained strands of fibrous material which usually failed to redissolve completely when warmed.

It will be noted in Table I that the heparin-precipitable material was not demonstrable until one hour after the injection of endotoxin; plasma samples obtained at 15 and 30 minutes were negative. The quantity of precipitate was much diminished in plasma obtained at 6 hours or later, and the majority were negative 24 hours after endotoxin. Precipitation was observed in a few plasmas from normal rabbits, as shown in Table I. In contrast to the rapidly appearing voluminous flocculation which occurred in plasma after an injection of endotoxin, the precipitates in normal plasma were usually small and did not appear

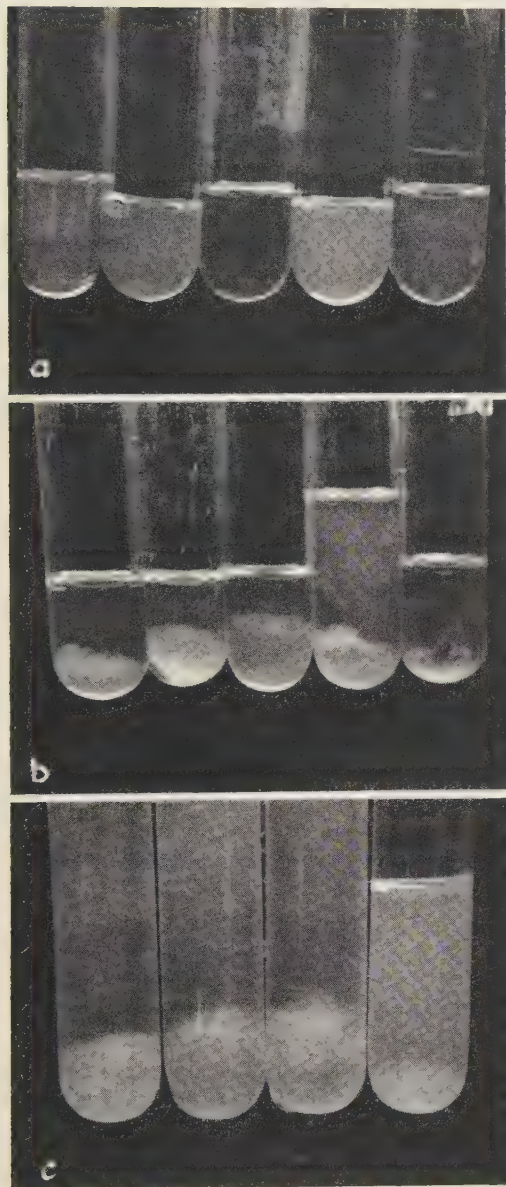


FIG. 1. Cold-precipitation by heparin in rabbit plasma. a. Plasma from 5 normal rabbits, containing 1 mg heparin/cc, photographed after chilling at 4°C for 2 hr. No visible precipitate. b. Heparinized plasma from the same rabbits shown above, obtained 2 hr after intrav. inj. of 2 cc 1-200 meningococcal endotoxin, photographed after chilling for 2 hr. Note flocculant precipitate in each tube. c. Heparinized plasma from 4 rabbits, obtained 2 hr after an intrav. inj. of 100 μ g of *S. marcescens* polysaccharide endotoxin, photographed after chilling for 2 hr.

degree of cold precipitation is indicated in Table I. It will be noted that the highest incidence of precipitation occurred in plasma containing 0.1 mg heparin per cc, while with 1 mg/cc precipitation failed to occur in several instances. Concentrations lower than 0.1 mg could not be tested for cold precipitation because of the frequent occurrence of clotting in plasma from animals injected with endotoxin.

Although cold-precipitation was not demonstrable in chilled citrated plasma obtained after endotoxin, it occurred promptly after the addition of heparin, in concentrations ranging from 1.0 to 0.01 mg/cc. Precipitation did not occur in citrated plasma from normal rabbits when the concentration of citrate was sufficiently high to maintain complete incoagulability. Thus, the addition of heparin to normal plasma containing 0.4% sodium citrate did not produce cold-precipitation. However, in normal plasma containing 0.2% citrate, which usually formed partial clots after several hours, cold-precipitation by heparin was frequently encountered. The phenomenon of cold-precipitation could not be brought about *in vitro* by the addition of endotoxin to heparinized or citrated plasma.

Relation of heparin-precipitable material to fibrinogen. The precipitated material was washed by repeated centrifugation at 4°C, and preliminary biochemical studies of its nature were made. The quantity of precipitate formed in plasma during 4 hours at 4°C, estimated by dry weight, ranged between 0.75 and 1.50 mg/cc; when estimated as protein by the biuret reaction the range was between 0.6 and 1.0 mg/cc. The following observations indicate that the material may be a protein related to or closely associated with fibrinogen. As mentioned above, it is present only in plasma and cannot be demonstrated in serum. Washed samples of the material, dissolved in warm saline, when subjected to paper electrophoresis were found to migrate in a manner similar to fibrinogen. Furthermore, the addition of thrombin to solutions of the material resulted in partial clotting. The occurrence of cold precipitation by heparin in normal plasma containing inadequate amounts of citrate is consistent with the view that the

until 4 hours or more after chilling. The effect of the concentration of heparin on the

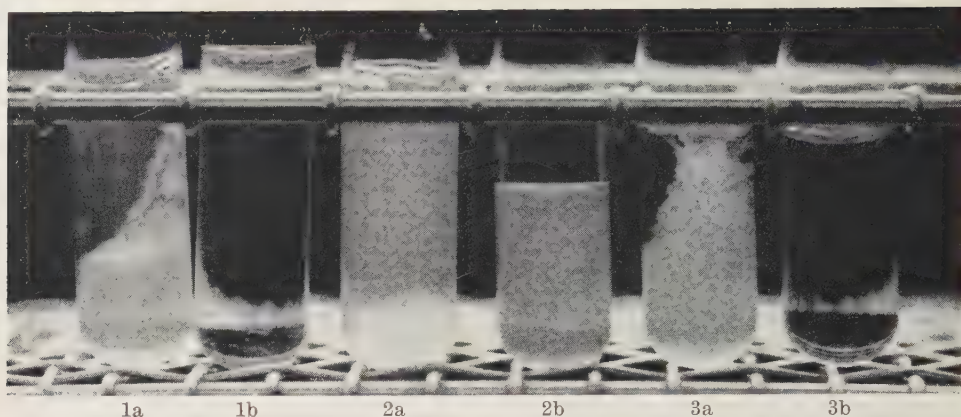


FIG. 2. Disappearance of heparin-precipitable protein after intrav. inj. of Liquoid. Three pairs of heparinized rabbit plasma are shown; 1a, 2a and 3a are plasmas obtained 3 hr after intrav. inj. of meningococcal endotoxin. Each rabbit then received 8 mg Liquoid intrav., and 1b, 2b and 3b are the heparinized plasmas obtained 10 min. after Liquoid. All photographed after chilling at 4°C for 18 hr.

precipitable material may represent a stage in the transition of fibrinogen to fibrin.

It is known that cold-insoluble globulins exist in crude fibrinogen preparations, sometimes referred to as "contractinogen"(4) or as the "non-clottable fraction"(5). Electrophoretically this component is closely associated with fibrinogen but is clottable by thrombin to a lesser extent or not at all. Although in the present study no cold-precipitation in plasma occurred in the absence of heparin, a cold-insoluble fraction was found in a commercial preparation of bovine fibrinogen (Armour). The degree of cold-precipitation from this sample was enhanced by the addition of heparin. A heparin precipitable fraction was noted in a sample of commercial human fibrinogen (Cutter). On the other hand, heparin did not cause precipitation of bovine fibrinogen from which the cold insoluble fraction had been removed by the procedure of Laki(6).

Disappearance of heparin-precipitable material after injecting "Liquoid". Liquoid (Hoffman-LaRoche) is a synthetic polymer, sodium polyanethol sulfonate, which possesses anticoagulant properties similar to those of heparin and, in addition, has the property of causing precipitation of fibrinogen from plasma *in vitro*(7,8). Repeated injections of large quantities of Liquoid, in rabbits, were shown by Tausman and Dreyfuss(9) to cause occlu-

sion of glomerular capillaries by homogeneous eosinophilic material. In this laboratory, the combined injection of Liquoid and small quantities of endotoxin has been found to produce the typical lesions of the generalized Shwartzman reaction(8). The addition of Liquoid to rabbit plasma containing the heparin-precipitable material resulted in prompt precipitation of this protein as well as fibrinogen. It was therefore of interest to determine whether the precipitable material was removed *in vivo* following an intravenous injection of Liquoid. Eight rabbits were injected with 2 cc of a 1-200 dilution of meningococcal endotoxin, and 2 hours later blood was obtained for heparinized plasma. They then received 8 mg Liquoid contained in 2 cc saline by vein, and were bled again 10 minutes later. In each instance, the chilled plasma taken before the injection of Liquoid contained large amounts of precipitate, while none was demonstrable in the plasma 10 minutes after Liquoid. Photographs illustrating the observation are shown in Fig. 2.

Heparin precipitable protein in human plasma. Heparinized plasma, containing 0.1 mg heparin per cc, was obtained from a group of 12 normal adult human beings, 6 human infants of 1 year or less, 6 children with acute rheumatic fever, and 1 child with acute rheumatoid arthritis. Aliquots of each were placed at 4°C and observed for precipitation. In

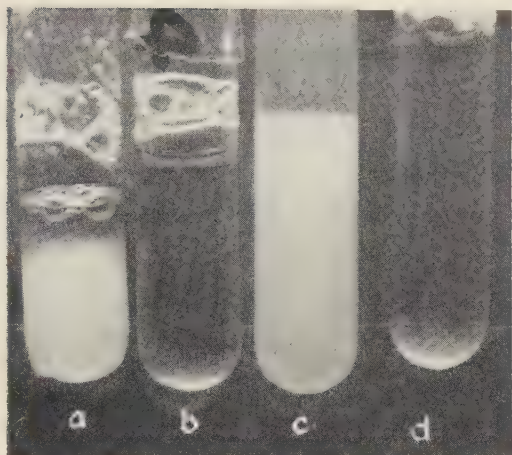


FIG. 3. Cold-precipitation by heparin in plasma of a patient with acute rheumatic fever. a. Chilled heparinized plasma (0.1 mg heparin/cc) obtained during first week of illness, prior to therapy. b. Plasma obtained 3 days after instituting salicylate treatment, at a time when patient was afebrile. c. Plasma 3 wk later, during exacerbation of the disease, while still receiving salicylate. Cortisone therapy was started after this specimen was obtained. d. Plasma after 3 days of cortisone treatment; patient afebrile at this time.

every instance, a flocculant, opaque precipitate formed within less than 4 hours after chilling, which was completely redissolved within a few minutes after rewarming the tubes. The amount of cold-precipitation appeared to be greater in the plasma of adults than in that of the normal infants. The heaviest precipitates occurred in the plasma of the patients with acute rheumatic disease, and precipitation became visible within a shorter time after chilling the plasma. The washed heparin-precipitable protein was examined by paper electrophoresis, and the migration of the material resembled that observed with a partially purified solution of human fibrinogen. In one patient with rheumatic fever, specimens of heparinized plasma were obtained at 4 different periods during his illness: at the time of admission to the hospital, several days after the administration of full therapeutic doses of salicylate, at the time of an exacerbation of the disease during salicylate therapy, and two days after subsequent treatment with cortisone. The cold-precipitation in these samples of plasma is shown in Fig. 3. It will be seen that the abundant precipitation present on admission decreased after salicylates, reappeared during

the exacerbation, and diminished again after cortisone. Further studies along similar lines are currently in progress and will be described in a later communication.

Discussion. A gelatinous, opaque protein precipitate occurs in chilled heparinized plasma obtained from rabbits between 1 and 6 hours after an intravenous injection of endotoxin derived from gram negative bacteria. The material is promptly redissolved on warming the plasma to 37°. It is not demonstrable in chilled citrated plasma until heparin is added.

The existence of the heparin-precipitable protein in plasma, but not in serum, suggests a possible relationship to fibrinogen. This receives further support from the observation that the washed, redissolved material migrates on paper electrophoresis in a manner similar to fibrinogen. Moreover, the washed protein is partially clottable by thrombin.

Other investigators have suggested that varying degrees of polymerization of fibrinogen may occur under certain conditions, resembling interrupted stages in the conversion of fibrinogen to fibrin(10). It is conceivable that the material under study may represent such an alteration in fibrinogen, with the formation of molecular aggregates capable of combining in an unstable complex with heparin.

The heparin-precipitable protein is to be differentiated from the cold-insoluble "non-clottable" fraction known to be present in crude fibrinogen preparations, since no precipitation occurs in chilled plasma in the absence of heparin. However, it should be noted that cold-precipitation of this fraction in bovine and human fibrinogen has been found in this laboratory to be augmented by heparin.

The mechanism by which heparin causes cold-precipitation is not clear. Walton(11) has shown that dextran sulfate preparations of large molecular size, with anticoagulant properties similar to those of heparin, cause reversible precipitation of fibrinogen at neutral pH by the formation of a complex, perhaps by coacervation. This investigator also found that heparin prevented precipitation by dextran sulfate, indicating a competitive affinity

of the two acidic polymers for fibrinogen. It is possible that a similar affinity may be responsible for cold-precipitation by heparin under conditions of increased molecular aggregation or polymerization of fibrinogen.

The possibility that the heparin-precipitable protein may be a precursor for the fibrinoid-like material which occludes the glomerular capillaries in the generalized Schwartzman reaction is under investigation. It is of interest that Liquoid, which precipitates the protein from plasma *in vitro*, produces the lesions of the generalized Schwartzman reaction when injected by vein in combination with endotoxin (9). Similar results, to be described elsewhere, have been obtained with combination of intravenous endotoxin and dextran sulfate of large molecular size. The observation that the heparin-precipitable protein disappears from the blood within 10 minutes after an injection of Liquoid is consistent with the view that it may be precipitated by Liquoid *in vivo*.

It is not known whether the heparin-precipitable protein in rabbit plasma is the same component as that which exists in normal human plasma. Preliminary paper electrophoretic studies of the latter material suggests a similar close relationship to fibrinogen. The marked increase in precipitable material observed in the plasma of children with acute rheumatic disease, and the diminution which was observed in one patient during salicylate and cortisone therapy suggest that a qualitative alteration in fibrinogen may have occurred in association with the disease. Further investigations of the protein in human plasma are in progress.

Summary. 1. Cold-precipitation of a mass of gelatinous protein material is produced by heparin in plasma obtained from rabbits between one and 6 hours after an injection of endotoxin derived from gram negative bac-

teria. The precipitate is redissolved on warming the plasma. It is demonstrable within 15-30 minutes after chilling heparinized plasma, or citrated plasma to which heparin has been added. 2. The heparin-precipitable protein is not demonstrable in serum. Its possible relationship to fibrinogen is indicated by similar migration in paper electrophoresis, and by the fact that it is partially clottable by thrombin. 3. The material disappears from the blood within 10 minutes after an intravenous injection of Liquoid (Na polyanethol sulfonate), an acidic polymer known to be capable of precipitating fibrinogen. 4. A heparin-precipitable protein of similar appearance is present in normal human plasma, and is much increased in the plasma of patients with acute rheumatic disease. In one patient with rheumatic fever, the amount of precipitate diminished during salicylate and cortisone therapy.

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Cultivation and Modification of Infectious Canine Hepatitis Virus in Roller Tube Cultures of Dog Kidney. (21242)

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The observation that multiplication of poliomyelitis viruses in tissue culture is accompanied by certain cytologic changes which are inhibited specifically by immune serum (1) has opened a broad field of study for the further application of tissue culture technics. In the study of certain highly host specific virus diseases of domestic animals research has been limited by the expense and necessity of providing both susceptible animals and suitable isolation quarters. Since infectious canine hepatitis (ICH) was described by Rubarth(2), investigations in this country have shown the disease to be of major importance among the canine population. Diagnosis is often difficult, since most frequently the only signs of disease are highly elevated temperature and leukopenia. Although the disease terminates fatally in only about 10% of the cases, there is a high carrier rate among recovered animals, the virus being excreted in the urine for as long as 161 days after infection(3). While recovery from infection results in lifelong immunity, immunization with the available inactivated tissue vaccines results in only a short-lived immunity.

It is the purpose of this paper to describe the cultivation and modification of ICH virus and a quantitative neutralization test for the diagnosis of this disease.

Materials and methods. Tissue cultures. Roller tube cultures were used throughout these experiments although the method of preparation varied. Up to the 48th passage of ICH, cultures of kidney explants were prepared by the plasma clot method described by Enders and his associates(4,5). Subsequent cultures were prepared by the simpler method of trypsinization of the minced kidney cortex used by Dulbecco and Vogt(6). A 0.2% suspension of washed cells was made in the nutrient fluid, which consisted of 8 parts Earle's-Simms solution, one part 5% lactalbumin, one part inactivated horse serum. Two ml of this suspension adjusted to pH

7.6-7.8 were placed in roller tubes and incubated stationary at 35°C until solid sheets of epithelial cells had formed on the walls of the tubes. The nutrient fluids were then renewed and the tubes were inoculated and placed in roller drums for further incubation. For the initial experiments kidneys were obtained from 2-4 week old pigs and for the remainder from 2-4 week old puppies from ICH and distemper immune bitches. All dogs used for virus inoculation were 2-5 months of age and were obtained from ICH susceptible litters.

Experimental. Propagation of the virus. During the course of experiments involving the use of pig kidney cultures, attempts were made to adapt ICH virus to this tissue. A 20% suspension of dog liver infected with ICH was inoculated into each of several tubes containing pig kidney explants. Serial transfers of 0.2 ml undiluted tissue culture fluid were made at 4-day intervals. No effects of the virus on the cells were noted in any tubes at any passage level. However, the 5th passage material when diluted 10^{-3} and inoculated in 0.2 ml amounts subcutaneously in 2 dogs resulted in the death of one animal with typical gross pathological lesions of ICH and a febrile response in the other dog. This animal, on recovery, was found to be immune to challenge with the original infected dog liver material. This tissue culture fluid represented a dilution of $10^{-8.7}$ of the original material which had a titer in dogs of only $10^{-6.5}$ when one ml was injected intravenously. At the 7th passage level in pig kidney the undiluted fluid inoculated into the 2 dogs resulted in the death of both. However, at the 8th passage ICH virus was no longer present. Cultures were then prepared from spleen and whole kidney of 2-week-old puppies. These were inoculated with 0.2 ml of undiluted fluid from the 3rd pig kidney passage. On the 6th day of inoculation only the epithelial cells in kidney cultures showed marked cytologic changes. The cells were rounded and highly refractile,

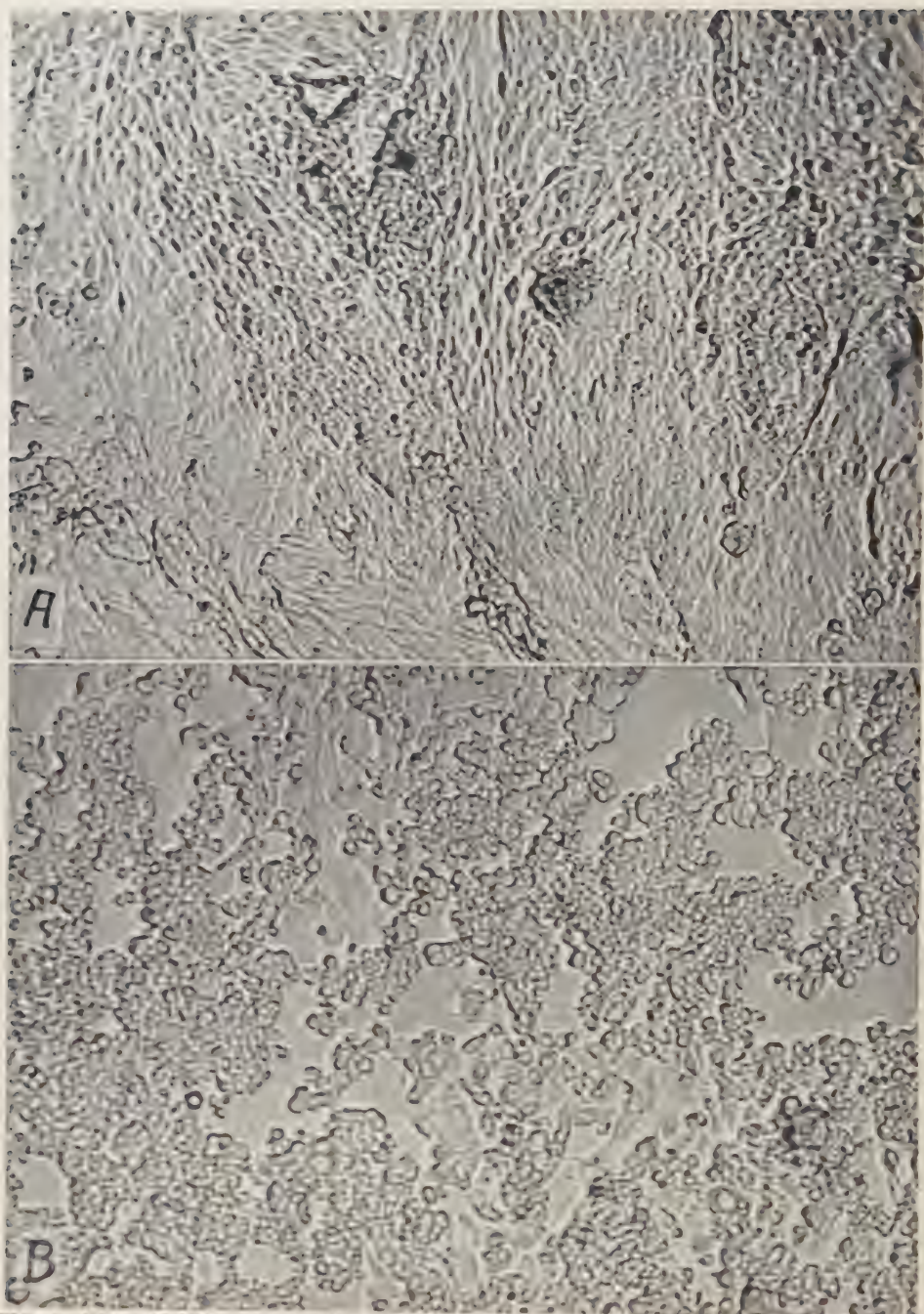


FIG. 1. Effect of ICH virus on roller tube cultures of dog kidney. (A) Uninoculated control tube showing clear sheet of epithelial cells. (B) Two days after inoculation with 10^4 TCID₅₀ of ICH virus, cytologic changes are evident.

The epithelial sheets had broken up and the cells were in small grape-like clusters. The fibroblasts in the same cultures were un-

affected and continued to grow even though all epithelial cells showed cytologic changes. The spleen cultures which contained only

fibroblasts remained normal throughout the 2-week observation period. The supernatant fluids from the kidney cultures were removed and serial passage of 0.2 ml of undiluted fluid was carried out. On subsequent passages the cytopathogenic effect occurred after 24-48 hours of incubation. Passages were made at 2-3 day intervals. The photomicrographs in Fig. 1 illustrate the effect of ICH virus on dog kidney cultures as seen 2 days after inoculation with undiluted tissue culture fluid.

Identification of the tissue culture virus was made at the 10th passage, at which time the titer of the virus was $10^{-5.6}$. Hyperimmune serum was prepared by inoculating a puppy with ICH virus known to be free of contamination. The dog developed a strong febrile reaction and 2 more injections of this virus were made 2 and 3 weeks after the original inoculation. The dog was bled and serum removed for neutralization tests. The serum had a neutralization index of 250,000+ and a 50% serum dilution endpoint of 1:4,096 against 400 tissue culture doses of virus, thus establishing the identity of the tissue culture virus with that of the original ICH virus.

Neutralization tests. At the 11th tissue culture passage a large pool of virus was prepared which had a titer of $10^{-6.2}$ in tissue culture and $10^{-7.5}$ in dogs. However, it was obvious at this time that a febrile reaction in dogs as an indication of ICH infection was unreliable, since any number of intercurrent infections might also give rise to a febrile reaction. The determination of antibody by the quantitative neutralization test offered a positive means of diagnosis and at the same time a method for testing the susceptibility of each dog in a litter before use in a test. All dogs were bled by cardiac puncture before inoculation and 2 and 3 weeks after inoculation. Decimal dilutions of sera were made in the tissue culture nutrient fluid, each dilution being mixed with an equal quantity of virus so that 0.2 ml of serum-virus mixture contained 100-320 TCID₅₀ of ICH virus. Incubation was carried out for 2 hours at 37°C after which time the 0.2 ml of each mixture was inoculated into each of 4 tubes. No sera were tested undiluted since it had been found that a large percentage of young dogs carried

TABLE I. Titration of Tissue Culture ICH Virus in 12 Dogs with Results of Serum Neutralization Tests.

Virus dilution	Clinical response	Reciprocal of 50% serum dilution endpoint against 100-320 TCID ₅₀ of ICH virus (pre-inoc. titer 1:4)	
		2 wk convalescent	3 wk convalescent
10^{-2}	F ₄	3160	3160
	F ₅	316	3160
10^{-3}	F ₄	2160	10000
	"	3160	4650
10^{-4}	"	2150	3200
	F ₅	3200	3200
10^{-5}	F ₃	3200	3200
	F ₄	1000	21500
10^{-6}	"	215	1000
	"	2150	3200
10^{-7}	F ₆ D ₁₂	—	—
	F ₆	3200	3200

F = Onset of febrile reaction on designated day.

D = Died 12 days post inoculation.

* Virus inoculated subcut. in 0.2 ml amounts.

passively transferred neutralizing antibody to ICH, insufficient to protect against the disease. All sera from each dog were tested simultaneously. Final readings were made at 5-6 days when the control titration showed 100-320 TCID₅₀ of virus to be present.

In Table I are summarized the data of a typical titration of ICH virus in dogs with the results of serum neutralization tests.

Although no endpoint was attained in this titration it is apparent that the dogs were highly susceptible to infection with the tissue culture virus and that high levels of antibody were reached as early as 8 days after the febrile response was first noted. The value of the neutralization tests for diagnostic purposes was emphasized by the fact that signs pathognomonic of the disease failed to develop in these dogs. Although the febrile reactions ranged from 39.8°C to 40.7°C they alone would not be sufficient to establish a diagnosis of ICH.

Modification of the virus. Serial passage of ICH was maintained over a period of several months. Occasional inoculation of dogs with undiluted virus was carried out to test for virulence and to check on the specificity of the cytopathogenic effect. Each of 30 dogs inoculated with virus from various tissue culture passages up to the 40th developed signs

TABLE II. Results of Passage of ICH Virus in Tissue Culture Showing Apparent Loss of Virulence for Dogs without Loss of Antigenicity.

Virus inoculum*	No. of dogs inoculated	No. showing clinical signs		% mortality	No. showing rise in neutralizing antibody titers†
		Fever	Corneal opacity		
TC #10 to 40	30	30	5	24‡	23
TC #51 to 70	13‡	1	0	0	13

* All dogs were inoculated subcut. with 0.2 ml containing 10^6 to 10^8 TCID₅₀ of virus.

† All sera had pre-inoculation titers of $<1:4$ and all showed at least 100-fold rise in antibody titer.

‡ Litter mates of these dogs when inoculated with virulent ICH virus developed definite signs of disease.

§ Diagnosis of ICH established post mortem either by presence of intranuclear inclusions or recovery of virus from liver and typical gross pathological lesions of ICH.

of disease. Only one of 13 dogs inoculated with virus from the 51st to 70th passages developed a slight febrile reaction. All dogs which had recovered from infection by the tissue culture virus were routinely challenged with the original virus from dogs and found to be solidly immune. Dogs inoculated with virus from the 51st to 70th tissue culture passages and challenged one month after inoculation were also refractory. It is conceivable, however, that these dogs were immune prior to inoculation with the tissue culture virus, even though litter mates had been highly susceptible. Neutralization tests were therefore utilized to establish the immune status of these animals. In Table II are summarized the data obtained by inoculation with virus from various tissue culture passages. The data suggest that the virus underwent modification in its virulence without loss of ability to stimulate antibody formation.

Discussion. Numerous attempts in this laboratory to adapt ICH virus to chick embryos, suckling mice, ferrets and rabbits have been without success. Although there have been reports of successful adaptation to chick embryos(7), ferrets(8), and rabbits(9), these results remain to be confirmed. The cultivation of ICH virus in cultures of dog kidneys has been reported recently by Cabasso *et al.* (10).

In order to avoid supply and maintenance of large numbers of susceptible puppies in isolation quarters, attempts were made to cultivate the virus in the tissues of the natural host. The virus proved readily adaptable to growth in kidney explants and also produced

readily recognizable cytologic changes. Furthermore, it was found that kidney cortex from 2-4 week old puppies had an extremely fast rate of growth, producing large sheets of epithelial cells in 3 to 4 days, in contrast to the slow rate of growth in cultures from older dogs. Since the puppies were obtained from bitches which had been recently hyperimmunized against ICH and canine distemper, the danger of accidental contamination with, and subsequent passage of, either of these viruses was minimized. In addition, kidneys from dogs of this age could be trypsinized more readily than those from older dogs. More than 400 tubes have been made from one pair of kidneys, all of which showed good, uniform growth.

The neutralization test in tissue culture has proven to be a most useful and sensitive diagnostic aid. All of 30 dogs whose sera had a pre-inoculation titer of 1:4 or less were shown to be susceptible to infection as indicated both by signs of disease and development of high titers of neutralizing antibody. On the other hand, 10 dogs which had pre-inoculation titers ranging from 1:1000-1:32000 were completely resistant to infection.

Up to the 40th passage of ICH the virus was highly virulent, producing a febrile reaction in all dogs inoculated, corneal opacity in 37% and death in 24%. However, at the 51st passage and through the 70th, the virus appeared to undergo a striking change in virulence. It no longer seemed capable of causing death or corneal opacity and produced a febrile reaction in only one dog. The immunizing properties of the virus remained un-

changed. At this point the value of the neutralization test for diagnostic purposes was further emphasized. After modification had occurred it was possible only by demonstrating a rise in antibody titer to prove that the dogs had been infected. However, diagnosis of ICH has also been made on numerous occasions by direct isolations of the virus in tissue cultures, either from a single drop of blood obtained during the febrile period; or after death from the livers of infected dogs(11). Thus, as has already been established for poliomyelitis, diagnosis can be made either directly by virus isolation or indirectly by demonstrating increase in antibody titer.

Recently, a comparison was made between virus from the 74th tissue culture passage and virulent ICH virus. There were 6 dogs in each group with an equal distribution of litter mates in each group. None of the dogs inoculated with virus from the 74th tissue culture passage developed either a febrile reaction or a leukopenia. Five of the dogs inoculated with virulent ICH virus died and had typical post mortem lesions of ICH, while the 6th dog developed a strong febrile reaction. All of the vaccinates developed a high titer of neutralizing antibody and resisted challenge with the virulent virus.

Summary. 1. Propagation of ICH virus

with the production of distinctive cytologic changes has been demonstrated in roller tube cultures of dog kidney. 2. After 51 serial passages the virus apparently had lost its virulence for dogs, although producing a solid immunity. 3. A practical neutralization test for the diagnosis of ICH has been described.

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Effects of Tumbling Trauma, Scalding and Hemorrhage on Rat Tissue Non-Protein Sulfhydryl.* (21243)

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In a preceding publication(1), 2 of us have reported that scalding, tourniquet trauma and exposure to severe cold for 6 hours all induced in the mouse a marked decrease in liver non-protein sulfhydryl (-SH) concentration. The present paper deals with the effects in the rat of tumbling trauma and scalding on non-pro-

tein -SH concentration in the blood, liver, and certain other tissues. Data on liver -SH values obtained for rats subjected to severe hemorrhage are also presented.

Materials and methods. *Chemical estimations.* All distilled water employed in the present experiments was freed of heavy metal ions capable of binding -SH by passing ordinary distilled water through a La Motte Filtration column. -SH estimations for deprotein-

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TABLE I. Comparative Tissue Non-Protein Sulfhydryl (-SH) Values Obtained for: (A) Control Rats, (B) Rats Traumatized by Tumbling (400 Turns) in a Noble-Collip Drum, or by Scalding (75°C for 15 Seconds).

Exp.	Time of sacrifice after tumbling or scalding	No. of rats in group	Non-protein -SH values, as GSH equivalents, mg %, \pm S.E.*				
			Liver	Kidney	Heart	Blood	
						Original	Adjusted to "100% Hb"†
A	Controls	16	194 \pm 7	108 \pm 4.4	59.3 \pm 1.6	39.4 \pm 1.6	40.6 \pm 1.6
	Tumbled rats:						
	5 min.	10	166	101	64	44.4 \pm 1.4	43.0 \pm 2.0
	40 "	4	126	96	62	39.4	38.6
	3 hr	4	110	103	43	39.6	36.0
	6 "	4	163	96	55	38.9	39.7
B	Controls	9	214 \pm 7	108	64	39.0 \pm 1.4	44.0 \pm 1.6
	Scalded rats:						
	3 hr	4	170	76	45	48.5	41.9
	6 "	8	148 \pm 8	86	67	46.5 \pm 1.2	41.4 \pm 1.0
	18 "	1‡	137	124	63	47.8	42.3
C	Controls	12	200 \pm 11	126 \pm 2.3			
	Scalded rats:						
	3 hr	8	156 \pm 12	84 \pm 4.9			
	6 "	4	161	92			

P values for control vs. scalded rats of Exp. B: For liver, less than .001.

Idem

Exp. C: " " , .01; for kidney, less than .001.

* Stand. error values not available in some cases, due to analytical values having been secured by using extracts from tissues pooled from several rats in the Group.

† See text.

‡ Only 1 of 4 scalded rats survived to 18 hr.

ized tissue or blood extracts secured in the scalding and tumbling experiments were made by the nitroprusside method of Grunert and Phillips(2), adapted to the Fisher Electrophotometer by increasing the volumes of all reagent solutions employed by them by 2.5 times, and the final volume to 25ml. In our experiments this method has given satisfactory and reproducible optical density readings for glutathione and cysteine solutions, tissue and blood extracts, and such extracts to which glutathione had been added. It has the great advantage over the amperometric method(3), which we had previously used(1), of permitting performance of analyses at a much greater rate. Since stability of the color developed by nitroprusside in the presence of -SH is apparently dependent on having a very high salt concentration, and since many proteins are precipitated at such high salt concentrations, it does not appear likely that the nitroprusside method will be readily adapted to estimations of total and protein -SH, which may be made fairly readily by the amperometric method(1). *Deproteinized blood* extracts for -SH estimation by the nitroprusside

method were secured as follows: A one ml hypodermic syringe bearing a short 23 gauge needle was filled to the 0.10 mark with heparin in 0.9% NaCl at a concentration of 1000 U. S. P. units per ml. The test rat was etherized, the chest wall opened, and blood drawn from the heart into the syringe to the 1.0 ml mark. This heparinized blood was discharged into a 10 ml glass stoppered volumetric flask. Two hundredths ml of the blood was withdrawn from the flask with a Sahli pipette and added to 5.0 ml of 0.1 N HCl, for estimation of hemoglobin content of the blood by the acid hematin method, using a Fisher Electrohemometer. To the heparinized blood remaining in the 10 ml flask was added 4 ml of distilled water and a pinch of saponin. On completion of hemolysis 2.0 ml of 8% metaphosphoric acid were added. The flask was stoppered and thoroughly shaken. Three grams of crystalline NaCl were added and shaken to solution. Foam in the neck was carefully removed using a wooden applicator dipped in caprylic alcohol. Saturated NaCl solution was added to the 10 ml mark. The contents of the flask were thoroughly mixed and filtered

TABLE II. Effects of Hemorrhage on Rat Liver Sulfhydryl.

Exp.	Avg total bleed- ing vol as % of body wt	Hr between end of 2nd bleeding & sacrifice	No. of rats in group	Liver -SH as mg %, GSH equivalent, ± S.E.			Protein extracted, as % of liver wt	Protein -SH, as % of extracted protein
				Total -SH	Non-protein -SH	Protein -SH		
A	Control	—	11	518 ± 35	187 ± 5	331 ± 35	17.4 ± 1.8	.20
	3.25	2	2	465	157	309	18.1	.18
	3.25	4	10	419 ± 40	142 ± 9	270 ± 40	16.7 ± 1.4	.17
B	Controls	—	10	615 ± 14	198 ± 11	417 ± 15	26.8 ± .4	.17
	2.77	24	10	645 ± 17	212 ± 7	433 ± 15	24.8 ± .6	.19
P values for control vs. bled rats of Exp. A, 4 hr after bleeding:				N.S.	.001	N.S.*	N.S.	
P values for controls of Exp. A (about 150 g body wt) vs. controls of Exp. B (older rats, about 210 g body wt):				.03	N.S.	.05	.001	

* N.S.—Difference not statistically significant.

through Whatman No. 1 paper into a test tube. A 5 ml aliquot of the filtrate, corresponding to 0.44 ml of rat blood, was employed for the nitroprusside optical density estimation.

In Table I, 2 sets of values are given for blood non-protein -SH: a) original values, b) values adjusted to blood containing hemoglobin at 100% of the "normal" value, arbitrarily placed at 15.6 g of hemoglobin per 100 ml of blood. Use of such a "normal" value makes it possible to allow for variations in non-protein -SH content of the blood due to variations in the mass of red cells subjected to analysis. The advisability of having "adjusted" values available for consideration is indicated by the finding of Peterson, Beatty and West(4) that whereas radiation was followed by marked changes in blood glutathione values, parallel changes also occurred in the hematocrit values, so that glutathione expressed as mg per 100 g of red cells was relatively little changed. *Deproteinized tissue* extracts for -SH estimation by the nitroprusside method were secured as follows: Immediately after the blood had been removed from the heart, each tissue for which an analysis was desired was excised, frozen on dry ice, wrapped in tin foil and held in a deep freeze to time of analysis, but not more than 24 hours after sacrifice. The frozen tissue was ground in a large mortar with sand and ice-cold 3% metaphosphoric acid, 4 ml per gram of tissue. Crystalline NaCl, 1.5 g per gram of tissue, was added and ground to solution in tissue

brei. Finally, 2% metaphosphoric acid saturated with NaCl was added in the amount of 14.5 ml per gram of tissue. The final mixture was filtered through Whatman No. 1 paper to give a clear filtrate. Concentration of non-protein -SH in the tissue has been estimated by multiplying the concentration found in the tissue extract by 20. In the *hemorrhage experiments* (Table II), the liver extracts were secured essentially as described previously(1), and the -SH estimations were made by the amperometric method(1,3). The values for *protein -SH as per cent of the protein*, shown in the final column of Table II, were secured as follows: Each protein -SH value, expressed as glutathione equivalent, was converted to -SH *per se*. This value was then used in conjunction with the corresponding extractable liver protein value, as estimated by the method of Robinson and Hogden(5), to calculate the term *protein -SH as per cent of protein*.

Animals and treatment. Carworth Farms strain male rats, fed on Rockland rat food pellets, were employed. Approximate average body weights for these rats were as follows: Exp. A of Table I, 240 g; Exp. B of Table I, 320 g; Exp. C of Table I, 270 g; Exp. A of Table II, 150 g; Exp. B of Table II, 210 g. *I. Tumbling trauma:* 400 turns (800 tumbles) were administered, using an apparatus modeled on that of Noble and Collip(6), but having a fixed rotation rate of 41 turns per minute. Preparation of rats for tumbling, and their

appearance and behavior after tumbling, differed in no way from the descriptions of Noble and Collip. For data obtained, see Table I, Exp. A. II. *Scalding*: The method described by Rosenthal(7) was employed. Each test rat was etherized and scalded to the neck in water at 75°C for 15 sec. For data obtained, see Table I, Experiments B and C. III. *Hemorrhage*: Each rat was etherized and bled from the tail by the method described for the mouse by Tabor, Kabat and Rosenthal(8). After completion of the second hemorrhage, the rats were returned to cages containing food and water. The amounts of blood secured by us, expressed as per cent of the body weight, have been appreciably less than the amounts which Tabor *et al* were able to secure from mice. They removed blood in a total amount of 4.5% of the body weight, in 2 equal bleedings an hour apart. While we were successful with each rat in removing blood in the amount of 2.25% of the body weight in the first bleeding, the second bleeding did not in any case yield an amount of blood exceeding 1.25% of the body weight. The amount yielded by rats of over 200 g body weight was appreciably less, in ml per 100 g of body weight, than that yielded by rats of about 150 g body weight. Of 24 rats subjected to hemorrhage, 2 died as a result of the first bleeding. The average total amounts of blood removed are indicated for the various groups of surviving bled rats in Table II. Rats subjected to hemorrhage and sacrificed at 2 or at 4 hours after completion of the second bleeding exhibited dyspnea, prostration and apathy at time of sacrifice. None of this symptomology was exhibited at 24 hours after completion of hemorrhage.

Results. I. *Tumble experiments* (Table I, Exp. A): The liver non-protein -SH values secured for tumbled rats were all less than the average liver non-protein -SH value obtained for control rats. This difference was most pronounced for tumbled rats sacrificed 3 hours after completion of tumbling. Other tissues analyzed (kidney, heart, skeletal muscle, blood) showed relatively little change in non-protein -SH, with the possible exception of heart at 3 hours after tumbling. II. *Scald experiments* (Table I, Exp. B and C):

Rats sacrificed at 3 and at 6 hours after scalding exhibited significantly lower non-protein -SH values for both liver and kidney than did control rats. Spleen non-protein -SH values were practically identical for control and scalded rats. Heart non-protein -SH was apparently decreased at 3 hours after severe scalding. At both 3 and 6 hours after scalding a considerable increase in blood hemoglobin had occurred, reflecting no doubt a corresponding hemoconcentration. There was also a considerable increase in the blood non-protein -SH. However, on adjustment of all bloods to a "100% Hb" value, the blood non-protein -SH values of control and scalded rats became statistically indistinguishable. III. *Hemorrhage experiments* (Table II): By 4 hours after the second hemorrhage a significant decrease in the liver non-protein -SH concentration had occurred (Exp. A). At 24 hours after hemorrhage, control and bled rats exhibited indistinguishable liver non-protein -SH values. The data of Table II indicate that no significant alteration in extractable liver protein or protein -SH was induced by the hemorrhage. More protein and protein -SH were extracted from the livers of the larger, older rats of Exp. B than from the livers of the younger rats of Exp. A. It is interesting to note that, within the limits set by the accuracy of the analytical methods employed, a fairly constant value was obtained for the factor *protein -SH as per cent of the extractable liver protein* (last column of Table II).

Discussion. A wide variety of procedures has been developed for producing in experimental animals a condition resembling clinical shock(9). From data presented in this paper and a preceding publication(1) it is apparent that in both the mouse and the rat the use of such procedures has to date invariably resulted within a few hours in a significant decrease in concentration of liver non-protein -SH compounds. Tests made to date have failed to reveal appreciable increases in non-protein -SH of tissues other than the liver, accompanying the significant decreases noted in the liver. Tissues of tumbled or scalded rats examined have included kidney, blood, heart, spleen and skeletal muscle. It would appear, therefore, that decreases in liver non-protein

-SH occurring in severely injured rats (and mice) are not brought about by the transfer to, and storage of, such compounds in unaltered chemical form in tissues other than the liver.

Leaf and Neuberger(10) and Edwards and Westerfeld(11) have reported that by 24 hours after rats were placed on a low protein diet, a considerable decrease had occurred in the concentration of glutathione in the liver. Since animals subjected by us to scalding, trauma, or hemorrhage did not eat for several hours thereafter, it might be postulated that the low liver non-protein -SH values observed by us were due to protein deprivation. However, the changes observed by us have been most pronounced at three and six hours after infliction of injury or hemorrhage, whereas in the experiments of Leaf and Neuberger rats deliberately starved did not exhibit any appreciable decrease in liver glutathione concentration even at 24 hours after the beginning of the starvation.

It is hoped that future experimentation will elucidate some of the metabolic and/or hormonal mechanisms responsible for the decreases in concentration of non-protein -SH compounds which occur in certain tissues, especially the liver, following severe injury.

Summary. 1. Significant decreases in concentration of non-protein -SH were noted in rat liver following tumbling trauma, and in rat liver and kidney following severe scalding.

2. In analyses confined to the liver, a significant decrease in liver non-protein -SH was noted following severe hemorrhage. No appreciable change was noted in either liver protein -SH or extractable liver protein. 3. Blood non-protein -SH concentrations were practically identical for control and tumbled rats. Scalded rats exhibited a moderate increase in concentration of blood non-protein -SH, paralleling the hemoconcentration.

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Activation of Bovine Plasminogen by Trypsin.*† (21244)

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Following the early observation that chloroform treatment increased the proteolytic activity of serum(1), chloroform was found to function by reacting with a protease inhibitor, thus allowing an autocatalytic type activation

of the protease(2). The inactive zymogen of this protease system has been named plasminogen (profibrinolysin) and the active enzyme, plasmin (fibrinolysin). Plasmin functions in the blood coagulation scheme by splitting fibrinogen and fibrin to smaller fragments. Since chloroform, first used to activate plasminogen, is foreign to a biological system, attempts have been made to find biological

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activators of plasminogen. Activators of human plasminogen have been found in streptokinase(3-6), staphylokinase(7,8), various mammalian tissues(3,5,9), human urine(10), human blood(6,11), and trypsin(8,12,19). Activators of dog plasminogen include staphylokinase(5,7,13), various mammalian tissues(5), dog serum(14), but not dog plasmin(5,8,13,14), and trypsin(8). Bovine plasminogen is not activated by bacterial kinases(5,7), but is activated by kinases found in human milk(15), human urine(10), human blood(6,11), lung tissue of several species(5,16), pig heart(3,17,18), and trypsin(12,16).

Trypsin can activate bovine prothrombin(20) as well as plasminogen and thus appears to function as a kinase for 2 important zymogens of the blood. In the present report trypsin is compared with other proteases and with chloroform as an activator of bovine plasminogen. Several of the bovine plasmins were partially purified and obtained in lyophilized form.

Methods and materials. Crude plasminogen.

Beef plasma was obtained frozen from a commercial source or prepared from fresh blood. In either case the blood was collected into oxalate solution(21) and centrifuged. The oxalated plasma was converted to serum by stirring 30 ml of 5.3% calcium chloride solution (added dropwise) into a liter of oxalated plasma. Fibrin precipitated in strands and stirring was continued for 30 minutes(22). The fibrin was removed on cheesecloth and the resulting serum was frozen in plastic containers at -20°C . Crude plasminogen was obtained from thawed serum by fractionation with ammonium sulfate at 0°C . Two hundred ml of ammonium sulfate solution (saturated at 25°C) was added dropwise to a chilled mixture containing 500 ml serum and 300 ml distilled water giving a solution of 0.82 M ammonium sulfate. If a precipitate formed, it was removed by centrifugation and discarded. Eighty grams of solid ammonium sulfate were then dialyzed into the chilled serum solution. After equilibrium, the contents of the dialysis tubing were added with stirring to the serum solution giving a final concentration of 1.38 M ammonium sulfate.

The solution was stored overnight at 5°C . Following centrifugation the supernatant was discarded and the precipitate was taken up in distilled water, yielding approximately one-fifth of the original serum volume. This ammonium sulfate fraction was frozen until further use. Upon thawing, 100 ml of this fraction was mixed with 20 ml of pH 7.2 imidazole buffer(23). The buffered product was called crude plasminogen solution. These procedures were based in part on those of Loomis *et al.*(22). In our work, freezing the serum and the ammonium sulfate fraction was convenient, and in addition, yielded a more active product than could be prepared without these freezing steps. The frozen products were stable for as long as 4 months. *Activation to plasmin.* To cause an autocatalytic type of activation, or to increase the efficiency of a protease activator, chloroform was used as follows: 100 ml of crude plasminogen solution (pH 7.2) was mixed with 20 ml reagent grade chloroform in a separatory funnel and the mixture shaken intermittently for an hour at room temperature. The solvent layer was then drawn off, and excess chloroform and denatured protein removed in an angle centrifuge. In the chloroform treatment, the critical factor appears to be adequate mixing of chloroform and crude plasminogen during the one-hour period. After such treatment, the chloroform may remain in contact with the supernatant liquid, be drawn off, or removed with denatured protein by centrifugation, with no effect on the activation. Procedures used in chloroform treatment were based in part on those of other workers(22,24). The chloroform-treated crude plasminogen solution was activated to crude plasmin solution at 25°C either slowly with no further treatment, or rapidly with addition of a protease activator. Trypsin, chymotrypsin, ficin, pancreatin, papain, and plasmin were tested for their ability to activate the plasminogen solution by adding them in the dry form. In the routine procedure with a protease activator the zero time fibrinolytic activity was measured just after the addition of the protease. This zero time activity, due to the protease activator, was subtracted from the total fibrinolytic activity obtained at later

time intervals as a measure of the plasmin produced. *Plasmin assay.* Assays were conducted by the method of Guest *et al.* (25), who define one fibrinolytic unit as the amount of protease activity which will completely lyse one cc of a standardized fibrin clot in 120 seconds at 28°C (26). *Plasmin purification.* The maximally activated crude plasminogen solutions were partially purified by 40-fold dilution with distilled water and precipitation at pH 5.5 with 0.1 *N* H₂SO₄. The preparation was stored overnight at 5°C and the supernatant decanted. The precipitate was centrifuged and the supernatant rejected. The plasmin precipitate was taken up in 0.066 *M* phosphate buffer, pH 7.5, to approximately one-tenth of the original serum volume and clarified by centrifugation. The procedure of isoelectric precipitation was repeated and the purified plasmin was again taken up in the phosphate buffer, shell frozen and lyophilized. These procedures were modified from those presented by other workers (22,24,27). Portions of the lyophilized plasmins were redissolved in phosphate buffer (10 mg/ml) and, after 20-30 minutes, were assayed for units of plasmin (fibrinolytic) activity. The total fibrinolytic activity of the dry products was assumed to be plasmin activity, since the plasmin was precipitated twice at pH 5.5 and trypsin was very soluble at this pH. Total nitrogen of the samples was determined by the micro-Kjeldahl method (28).

Results. The activation of crude plasminogen to plasmin was followed by the fibrinolytic assay method over a period of time to obtain the maximum increase in activity. This increase was recorded as plasmin activity. Such a method has been used previously by other workers who activated human, dog, and bovine plasminogen with trypsin (8,12,16). Although the fibrinolytic assay method does not distinguish between trypsin and plasmin, the increased fibrinolytic activity observed is most easily explained by attributing it to the trypsin activation of crude plasminogen.

In our activation studies, trypsin caused activation of crude bovine plasminogen as reported previously (12,16). However, data (Table I) show that sufficient trypsin must be present to overcome the trypsin inhibitor in

TABLE I. Activation of Crude Bovine Plasminogen by Trypsin.

Trypsin conc. (mg/ml crude plasminogen sol.)	Zero time activ- ity† (fibrinolytic units/ml crude plasminogen sol.)	Time to max activation (hr)	Plasmin produced (increased units/ ml crude plasmin- ogen sol)
.2	0	—	0 (21 hr)
.4	4.5	2	20
.6	15	1	32
.8	32	1/2	29
1.0	28	1/4	31
1.1	27	1/4	28
1.2	45	1/4	24
1.4	57	< 1/4	1 (15 min.)
.05*	0	4 1/2	17
.10*	0.7	4 1/2	21
.15*	5.6	2	24
.2 *	6.2	1 1/4	24
.3 *	16	1/2	28
.4 *	11	1/2	25
.5 *	15	1/2	23
.6 * \	16	1/4	22
.8 *	32	1/4	25
1.0 *	72	< 1/4	0 (15 min.)

* After chloroform treatment of crude plasminogen solution.

† Due to trypsin activator.

the crude plasminogen, or plasmin will not be produced. Jacobsson (19) observed a similar trypsin inhibitor in his studies with human plasminogen. Our data show that if sufficient trypsin is present to cause maximum activation, increased trypsin decreases the time for the conversion; the data suggest that trypsin functions enzymatically in this conversion. With excessive levels of trypsin, the increase in plasmin activity is not observed (Table I). In these cases the conversion may be so rapid that the zero time activity measurement includes both trypsin and plasmin activity.

If the solution of crude plasminogen is first treated with chloroform, the amount of trypsin required for maximum activation is reduced (Table I). Thus, either chloroform or excess trypsin eliminates the blocking effect of trypsin inhibitor in the crude plasminogen. Since trypsin activation was more efficient with the chloroform-treated crude plasminogen solution, this solution was used as the substrate in the testing of other proteases as activators. The proteases tested (chymotrypsin, ficin, pancreatin, papain, and partially purified bovine plasmin) were used in amounts

TABLE II. Activation of Crude Bovine Plasminogen by Various Activators.

Activator†	Conc. (mg/ml crude plasmin- ogen sol.)	Zero time ac- tivity‡ (fibrin- olytic units/ml crude plasmin- ogen sol.)	Time to max activation (hr)	Plasmin pro- duced (in- creased units/ ml crude plas- minogen sol.) (hr)
Chloroform	Saturated	0	100–120	19
Trypsin	1.0	17.3	6	88
Trypsin	.1*	2.5	6	62
Chymotrypsin	.1*	3.0	—	0 (6)
Ficin	1.0*	1.0	—	0 (6)
Pancreatin	5.0*	1.5	4	55
Papain	5.0*	1.0	—	0 (6)
Plasmin (chloroform activated)	25.0*	1.0	48	16

* After chloroform treatment of crude plasminogen solution.

† Source of activator: Chloroform—"Baker Analyzed" Reagent. Trypsin—Armour crystallized trypsin of bovine origin, not more than 50% MgSO_4 . Chymotrypsin—Crystallized 3 times. We wish to thank Mr. H. N. Wood and Dr. A. K. Balls for this preparation. Ficin—Bios Laboratory, Inc. Pancreatin—U.S.P. Nutritional Biochemicals Corp. Papain—Nutritional Biochemicals Corp. Plasmin—Prepared in the laboratory using chloroform activation.

‡ Due to enzyme activator.

which had approximately the same fibrinolytic activity per ml of chloroform-treated crude plasminogen solution (at zero time) as 0.1 mg of trypsin. When tested over a period of time, fibrinolytic (plasmin) activity increased when trypsin, pancreatin, or partially purified plasmin were used as activators, but did not increase when chymotrypsin, ficin, and papain were used (Table II). Chloroform alone was tested on this lot of crude plasminogen and produced approximately the same increased level of plasmin activity (19 units/ml) as did partially purified bovine plasmin (16 units/ml). Trypsin was the best activator tested.

The data in Tables I and II were obtained by similar methods, but are not directly comparable since different lots of crude plasminogen were used. Separate portions of a third lot of crude bovine plasminogen were activated by trypsin alone, trypsin following chloroform treatment, and chloroform treatment alone. In all cases the activation was followed to maximum activity, at which time the active plasmin was purified by isoelectric precipitation at pH 5.5, and lyophilized as described under *Methods*. The trypsin used for activation of plasminogen was very soluble at pH 5.5.

The lyophilized products were compared on the basis of purity and yield. The data in Table III show that the plasmin activity per

mg of nitrogen, and the plasmin yield per liter of serum were higher with both types of trypsin activation than with chloroform activation. It is interesting to note that the differences between chloroform-trypsin and trypsin activation are apparent in the crude solutions (Tables I and II) but are not apparent in the lyophilized plasmins (Table III).

When plasmin was lyophilized from phosphate buffer, pH 7.5, the dry preparations retained all of their activity when stored for 2 weeks in a dessicator *in vacuo* at -20°C . When stored at room temperature for 2 weeks in a screw-top bottle, however, only one-third of the plasmin activity was retained.

Summary. Crude bovine plasminogen was activated to plasmin by trypsin, by chloroform treatment which allowed an autocatalytic type activation, or by chloroform treatment followed by rapid activation with a low level of trypsin. Since the last method was an efficient one, other proteases were tried in the place of trypsin. Pancreatin and partially purified bovine plasmin also activated crude plasminogen but chymotrypsin, ficin, and papain did not. Trypsin was the best activator of the series tested. Partially purified plasmins activated by trypsin, chloroform, or chloroform-trypsin were lyophilized and compared with one another. The plasmins prepared by trypsin activation appeared to be more potent

TABLE III. Comparison of Dry Bovine Plasmins Prepared with Different Activators.

Activator	Trypsin conc. (mg/ml crude plasminogen sol.)	Time to max activation (hr)	Activity of plasmin† (units/mg N)	Yield of plas- min† (units/l serum)
Trypsin	1.0	1/4	5.0	1390
	1.0	1/4	5.5	1500
	.8	1/2	5.5	1340
Trypsin*	.4	1/2	4.5	1510
	.4	1/2	5.0	1640
	.2	3/4	6.0	2310
Chloroform	.0	300	.8	230
	.0	194	.6	225
	.0	194	.9	320

* After chloroform treatment of crude plasminogen solution.

† After separation from trypsin by ppt 2 times at pH 5.5.

than those prepared by chloroform activation alone.

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Stabilizing Effect of Amino Acids on Bacteriophage T1r* (21245)

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Burnet and McKie(1) reported the effect of certain ions on the stability of coli and staphylococcal bacteriophages. Sodium, potassium and ammonium salts increased the susceptibility to inactivation while small amounts of calcium, magnesium or barium salts reversed the influence of the first group. Later Adams(2) studied in detail the stability of the "T" series of coli-dysentery phages with particular emphasis upon phage T5. Essentially his results confirm the findings of Burnet and McKie.

In the course of experiments with T1r⁺ phage it was found that certain amino acids stabilized this bacteriophage as do calcium and magnesium ions under conditions similar to those of Burnet and McKie(1) and of Adams (2). The rapid inactivation of diluted phage in 0.15 N sodium chloride, distilled water or Davis-Mingioli medium(3) at 37°C was prevented. This finding is reported in the hope that it may be of interest in studies on factors affecting the stability of these agents.

Materials and methods. The T1r⁺ phage used in these experiments was obtained from Dr. Hotchkiss of the Rockefeller Institute. Stock phage was grown on *E. coli*, strain K-12, in the medium of Davis and Mingioli(3) with and without sodium citrate. At a temperature of 2-4°C phage stocks showed little loss of activity for 5 or 6 months. Phage assays were made on both *E. coli*, strain K-12 and strain B, using the agar layer technic as set forth by Adams(2). The plating medium was a tryptic digest of beef heart agar, 0.5% in sodium chloride. For other details see Table I. All glassware was cleaned with acid dichromate and liberally rinsed with tap and distilled water.

Results. Experiments designed to follow the reproduction of coli phage T1r⁺ on K-12

TABLE I. Effect of Calcium, Mg, and Mn Ions on Inactivation of Phage T1r⁺ at 37°C.

Diluent	Phage assay* at time		
	0	1 hr	3 hr
(0.5% glucose)			
† Davis medium (no citrate)	460	40	20
‡ Same + Ca ⁺⁺ (.001M)	404	70	28
+ Ca ⁺⁺ (.01 M)	490	361	320
+ Mg ⁺⁺ (.001M)	440	65	40
+ Mg ⁺⁺ (.01 M)	473	325	300
+ Mn ⁺⁺ (.001M)	458	73	30
+ Mn ⁺⁺ (.01 M)	420	45	35
Broth	465	378	347

* Phage assays are the No. of plaques formed on nutrient agar with *E. coli*, strain K-12 as indicator strain: (*E. coli*, strain B gives essentially the same values).

Each reported figure represents avg of triplicate experimental tubes. Final vol of each tube was 1 ml; of this a 0.1 ml sample was removed for plating after technic of Adams(2) and the phage of remaining 0.9 ml estimated on a second plate by same technic. This partition of sample was followed to insure a countable plate.

† All values expressed as final molar concentration.

‡ Expressed as soluble Ca⁺⁺ which remains after precipitation by phosphate in medium. (Estimated by calculation from solubility constants.) Acid salts were neutralized before addition.

employing a small inoculum of phage particles were complicated by the rapid loss of the inoculum at 37°C(4). This inactivation proved to be unrelated to the presence of bacteria and could be demonstrated when phage alone was incubated at 37°C in the medium of Davis and Mingioli, distilled water or .15 N sodium or potassium chloride. The rate of inactivation was essentially the same in each of these diluents.

Broth as the suspending medium preserved the infectivity of the phage as did synthetic medium to which calcium or magnesium ions were added at 0.01 M concentration (Table I). Results obtained on addition of calcium, magnesium or manganese to distilled water and to .15 N sodium chloride solution as the suspending fluid were not significantly different from those presented for synthetic medium in Table I.

Certain amino acids, purine and pyrimidine

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TABLE II. Amino Acids as Stabilizers of T1r⁺ Bacteriophage at 37°C.

Diluent	Phage assay at time		
	0	1 hr	3 hr
Davis medium (.5% glucose, no citrate)	620	40	7
* + DL-Threonine (.001M)	604	427	330
+ D- Threonine "	585	473	415
+ L- Threonine "	593	484	400
+ DL-Aspartic "	611	436	370
+ D- Aspartic "	603	450	410
+ L- Asparagine "	560	92	12
+ L- Glutamic "	564	500	463
+ DL-Serine "	580	380	345
+ DL-Homoserine "	625	430	370
+ Glycine "	623	53	8
+ DL-Valine "	577	74	32

* Final molar concentration for all compounds. The following compounds exerted no stabilizing effects: results essentially same as seen with glycine and valine (above): DL-Isoleucine, L-Leucine, DL-Methionine, DL-Norvaline, DL-Ethionine, DL-NH₂ Butyric acid, B-OH Butyric acid, L-Lysine, L-Arginine, Adenine, Cytosine, Cytidine, Cytidylic acid, Uridylic acid, Uracil, Guanine, Guanosine.

bases, nucleosides and nucleotides and B-OH butyric acid were tested singly for their effect upon phage stability. It was found that certain amino acids at a concentration of .001 M or .01 M did stabilize the phage while others and compounds of other classes did not (Table II). Glutamic and aspartic acids, serine, threonine and homoserine were effective stabilizers, while asparagine, lysine, arginine and B-OH butyric acid were not. It will be seen that either D- or L-threonine, DL- or D-aspartic acid preserved the infectivity of the phage.

When added together, calcium and aspartate at certain concentration ratios seem to counter-

TABLE III. Effect of Calcium plus Aspartate on Stability of T1r⁺ Phage at 37°C.

Diluent	Phage assay at time		
	0	1 hr	3 hr
Davis medium (.5% glucose, no citrate)	483	36	20
* + Ca ⁺⁺ .01M	434	311	297
+ DL-Aspartate .001M	462	277	233
+ Ca ⁺⁺ .01M + DL-Aspartate .00001M	450	302	286
+ Ca ⁺⁺ .01M + DL-Aspartate .0001M	430	233	182
+ Ca ⁺⁺ .01M + DL-Aspartate .001M	446	103	34

* Expressed as soluble Ca⁺⁺ which remains after precipitation by phosphate in medium. (Estimated by calculation from solubility constants.)

act each other. From the data in Table III it will be seen that the concentration of calcium ion sufficient to stabilize phage by itself fails to prevent inactivation in the presence of DL-aspartate, 0.001 M. Aspartate, .0001 M, also counteracts to a slight extent the stabilizing effect of calcium.

Discussion. The effect of the suspending medium upon the stability of bacteriophages of the T series has been studied by several investigators. Burnet and McKie(1) and Adams(2) have reported in some detail the relative instability of these phages in dilute solutions of sodium salts at 37°C and the protection of phage in this unfavorable circumstance by a variety of cations. Adams(2) found that in dilute sodium chloride the rate of inactivation of phage T5 was greatly decreased by the presence of .001 M concentrations of Ca, Mg, Ba, Sr, Mn, Co, Ni, Zn, Cd or Cu.

The instability of phage T1 has been mentioned by Puck(4) as a property apart from the reversible inactivation under particular conditions with which he dealt at length. The reversible inactivation was shown to take place on contact with bacteria after a period of preincubation of phage alone in buffer. The inactivation dealt with in the present study is not reversible by addition of cations or amino acids after preincubation of the phage and is unrelated to the presence of bacteria. It has been demonstrated to take place at 37°C in a synthetic medium with or without glucose or citrate, in distilled water or in .15 N sodium or potassium chloride. Although addition of calcium or magnesium in .01 M concentration results in increased stability of the phage, this effect could not be produced with manganese chloride or sulfate. No other metals were tried.

As effective as cations in making the phage more stable were the amino acids, threonine, serine, homoserine, aspartic acid and glutamic acid. These are all amino acids having two potentially ionized acidic groups at pH 7.0. Neither neutral nor basic amino acids could be demonstrated to increase the stability of phage. Indeed asparagine, in which one of the carboxyl groups of aspartic acid has been substituted with an amino group, is not effective. From this it might be suggested that two

acidic groups or the combinations -OH and -COOH on the amino acid are essential in stabilizing the bacteriophage.

The separate effects of calcium and aspartate are suppressed by combination of the two at certain levels. The binding of metals by amino acids is a well established fact and appears a reasonable explanation for the mutual suppression.

Summary. 1. T1r⁺ phage is rapidly and irreversibly inactivated at 37°C in distilled water, .15 N sodium or potassium chloride or in Davis medium. 2. Addition of calcium,

magnesium or the amino acids serine, threonine, homoserine, aspartate or glutamate results in stabilization of phage in the diluents mentioned above.

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Limits of Absorption of Orally Administered Vitamin B₁₂: Effect of Intrinsic Factor Sources.* (21246)

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Studies of patients having pernicious anemia(2,3) and of persons with total gastrectomy(4,5) have shown that when vit. B₁₂ is given orally in doses of 0.5 µg its absorption from the gastrointestinal tract is dependent upon the presence of a gastric substance, presumably the Castle-defined intrinsic factor(6). However, the role of intrinsic factor in facilitating absorption of vit. B₁₂ has not as yet been clarified and, moreover, the pattern of absorption of this vitamin in subjects with normal gastric secretion has not been defined.

In the present investigation, a study has been made of the absorption of vit. B₁₂ in individuals with clinically normal gastric function. Graded doses of Co⁶⁰-labeled vit. B₁₂[†] were administered orally and the total amount of radioactivity excreted in stool samples measured by methods previously reported(2,5).

Results. 0.5, 2, 5, and 10 µg amounts of labeled B₁₂ were given in a series of doses at

TABLE I. Fecal Excretion of Co⁶⁰ following Graded Doses of Co⁶⁰ Vit. B₁₂ Given to Normal Subjects.

Dose in µg	No. of tests	% Co ⁶⁰ excreted		µg vit. B ₁₂ absorbed (calculated)	
		Range	Avg	Range	Avg
.5	15	57-19	35	.22-.41	.34
2.0	12	69-30	48	.61-1.44	1.03
5.0	15	87-50	67	.73-2.50	1.65
10.0	5	93-70	84	.68-2.97	1.63

various time intervals to subjects who were either fasting or between meals. Each dose contained 0.110 µc of Co⁶⁰. No test was repeated with a given subject using the same dose, although most subjects received more than one dose level. It was found (Table I) that as the amount of administered vit. B₁₂ was increased, the per cent of Co⁶⁰ excreted in the stools increased. The amount of vit. B₁₂ presumed to be absorbed, a figure obtained by subtracting the apparent B₁₂ excreted from the total amount administered (Table I), was no greater with 10 µg than with a 5 µg dose, an average value of approximately 1.6 µg being the calculated absorption at both dose levels.

The effect of a test meal on vit. B₁₂ absorp-

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TABLE II. Effect of Test Meal on Fecal Excretion of Co⁶⁰ at Varying Doses of Co⁶⁰ Vitamin B₁₂.

Dose of Co ⁶⁰ B ₁₂ (μg)	% Co ⁶⁰ excreted	
	Fasting	With test meal
.5	42	19
	50	26
	52	20
	65	28
	31	27
	25	28
	27	25
5	74	86
	57	51
	78	67
	50	58

tion was then investigated. The meal, given as breakfast, contained no sources of vit. B₁₂. It was found (Table II) that after 0.5 μg of the vitamin was administered in 4 of 7 subjects, food decreased the excretion of Co⁶⁰ and it may be significant that the 3 in whom no effect of food was found excreted considerably less radioactive material when fasting than the other 4. These results suggest that secretion of intrinsic factor may be stimulated by food. However, in subjects given 5 μg vit. B₁₂ (Table II), no difference was obtained in Co⁶⁰ excreted in the fasted as compared with the fed state. It should be pointed out that in all these tests, stools were collected until radioactivity no longer appeared.

In further studies of the absorption of vit. B₁₂ at the 5 μg dose level, relatively large amounts of intrinsic factor were administered simultaneously. In 4 individuals who were given either gastric juice or intrinsic factor concentrate from hog stomach mucosa together with 5 μg vit. B₁₂ it was found that the addition of these substances did not enhance vitamin absorption (Table III). In 3 instances an inhibitory effect seems to have been obtained.

In additional experiments with 2 gastrectomized subjects, the effect of graded doses of the intrinsic factor sources mentioned above was compared at both the 0.5 μg and 5 μg of vit. B₁₂ dose levels (Table IV). When 0.5 μg B₁₂ was administered, increasing the amount of intrinsic factor increased the amount of B₁₂ absorbed. At 5 μg, absorption was also increased by intrinsic factor addition. However, a larger amount of intrinsic factor

supplement did not further increase B₁₂ absorption and the B₁₂ absorbed was within the range obtained in subjects with normal gastric function. Similar results were obtained when gastric juice was used as a source of intrinsic factor.

Glass and coworkers have reported (7) that in normal subjects the per cent of vit. B₁₂ taken up by the liver varies inversely with the oral dose when the vitamin is given in amounts of 0.5 to 50 μg. Hence the available evidence points to the extreme limitation of vit. B₁₂ absorption even in normal individuals.

In individuals with a functioning gastric mucosa, when the amount of vit. B₁₂ absorbed is not increased by a larger dose, gastric juice or hog mucosal extracts have no effect in increasing vitamin absorption. It would appear that here some factor other than intrinsic factor is operating to limit the passage of B₁₂ across the intestinal membrane. The results indicate that the materials used—gastric juice and hog stomach extracts—might actually inhibit B₁₂ absorption. An intrinsic factor concentrate has been shown to inhibit vit. B₁₂ absorption in rats also (8,9). The inhibitory effect observed in these various experiments is not necessarily due to the intrinsic factor *per se*, since the administered substances were not pure. In this regard, Wijmenga and coworkers (9) have isolated from combinations of hog mucosa extracts and crystalline vit. B₁₂ a cobalamin protein with a high molecular weight which does not by itself have hemopoietic activity. In the pres-

TABLE III. Co⁶⁰ Excretion following Administration of Graded Doses of Intrinsic Factor and Labeled Vit. B₁₂ to Normal Subjects.

Sub- ject	Dose		Co ⁶⁰ ex- creted, %	Vitamin B ₁₂ absorbed (calculated) μg
	Vit. B ₁₂ , μg	Intrinsic factor		
Ma	5		81	.94
	5	400 mg conc.*	91	.45
Mc	5		60	1.98
	5	<i>Idem</i> *	91	.45
Ha	5		57	2.15
	5		51	2.40
	5	150 ml gastric juice	90	.48
Ga	5		74	1.32
	5	200 mg conc.*	71	1.43

* Hog gastric mucosa fraction, kindly supplied by Dr. R. W. Heinle of Upjohn Co.

TABLE IV. Co⁶⁰ Excretion following Administration of Graded Doses of Intrinsic Factor and Labeled Vitamin B₁₂ to Patients with a Total Gastrectomy.

Subject	Dose		Co ⁶⁰ excreted, %	Vitamin B ₁₂ absorbed (calculated) μ g
	Vit. B ₁₂ , μ g	Intrinsic factor		
Mo	.5		100	0
	.5	2.5 mg conc.*	64	.18
	.5	5.0	28	.36
	5.0		91	.45
	5.0	200	65	1.74
	5.0	400	62	1.93
St	.5		95	.03
	.5	25.0 mg conc.†	74	.13
	.5	50.0	28	.36
	5.0		87	.65
	5.0	150 ml gastric juice	40	3.00
	5.0	300	38	3.10

* Supplied by Merek & Co., Inc.

† " " Upjohn Co.

ent experiments, the formation of a similar compound which is not readily absorbed might explain the inhibitory results and serve also as a basis for explaining the limitation of absorption observed when doses of the order of 5 to 10 μ g of B₁₂ alone were given. At this concentration of vitamin the cobalamin protein might be formed within the gastrointestinal tract.

Summary. The results show the extreme limitation of vit. B₁₂ absorption from the gastrointestinal tract of individuals with normal gastric function. Within the dose range used,

an upper level of absorption appears to have been reached which averages 1.6 μ g vit. B₁₂. This level is not increased by giving the vitamin with a test meal or in conjunction with intrinsic factor sources. In patients with a total gastrectomy, a graded response to intrinsic factor is obtained using 0.5 μ g vit. B₁₂ but at a 5 μ g level, increasing the amount of intrinsic factor does not increase B₁₂ absorption beyond levels obtained in normal subjects.

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Regeneration of Tissue Nonprotein Sulfhydryl Compounds in Rats After Exposure to Cold and Restraint.* (21247)

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It has been previously reported(1-4) that cold and restraint lower the concentration of

liver nonprotein sulfhydryl compounds. Since Waelsch and Rittenberg(5,6) and Block(7,8) reported a rapid turnover of liver glutathione, this study was done to determine if the regeneration of these sulfhydryl compounds would be equally fast after the concentration was lowered by exposure to cold and restraint. To observe the effect of caloric intake on the

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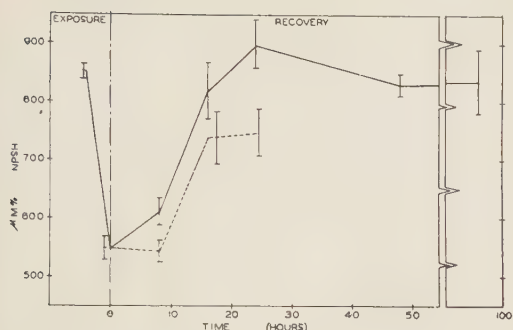


FIG. 1. Effect of fasting (hatched line) and non-fasting (solid line) on regeneration of liver non-protein sulfhydryl compounds in rat after exposure to cold and restraint. Stand. error of mean indicated by vertical lines on the curves.

rate of regeneration, food was withheld from some of the animals.

Methods and materials. Eighty-five female Sprague-Dawley rats weighing between 175 and 200 g were treated as follows: 10 animals were used as controls. The remaining 75 animals were exposed to cold and restraint, and body temperature drop was regulated to 5° per hour so that at the termination of a 4-hour exposure the body temperatures were approximately 18°C. Ten animals were sacrificed at the termination of this exposure period. The other 65 animals were divided into 2 categories. Twenty-one of the rats were given no food and groups of 7 were sacrificed at 8, 16, and 24 hours respectively. Forty-four of the rats were given food *ad libitum* and groups of 7 were sacrificed at 8 and 16 hours, and groups of 10 were sacrificed at 24, 48, and 96 hours respectively. The animals were not restrained during the recovery period during which time they were placed in individual laboratory cages. All of the animals were stunned with a blow on the head, decapitated to avoid congestion of the organs with blood, and the tissues taken immediately and frozen in powdered dry ice. The total non-protein sulfhydryl concentration (NPSH) of the liver and kidney was determined using a modification of the method of Benesch and Benesch (9).

Results. As seen in Fig. 1, regeneration of the lowered liver NPSH was virtually completed by 16 hours in the nonfasting animals. At 8 hours there was little regeneration of the

sulfhydryl compounds. In the fasting animals regeneration was somewhat slower in starting, and recovery of control liver levels was not complete in 24 hours.

As observed from Fig. 2, it appears that there was a small drop in the kidney NPSH as a result of exposure to cold and restraint. In both the fasting and nonfasting animals control levels were reestablished in less than 8 hours. In both of these categories there seemed to be an overcompensation so that there was an increase in kidney NPSH above the control values. In the nonfasting animals control values were reestablished again in 48 hours.

Discussion. The rate of regeneration of NPSH in the liver after exposure to cold and restraint was much less than the rate of loss during exposure to these stresses (Fig. 1). However, until normal body temperatures had been established (5 or 6 hours), maximum regeneration of sulfhydryl compounds would probably not be expected. It can be seen from Fig. 1 that there was no significant increase in the liver NPSH 8 hours after removal from the cold. Even at 16 hours recovery was not complete.

Since glutathione (GSH) comprises nearly all the NPSH in the liver (ergothionine, which comprises less than 10% of the NPSH, is not altered by cold and restraint(3)) it would be expected that the half life of NPSH would be approximately that of GSH. Waelsch and Rittenberg(5,6) suggested that the half life of liver GSH was 2 to 4 hours in normal ani-

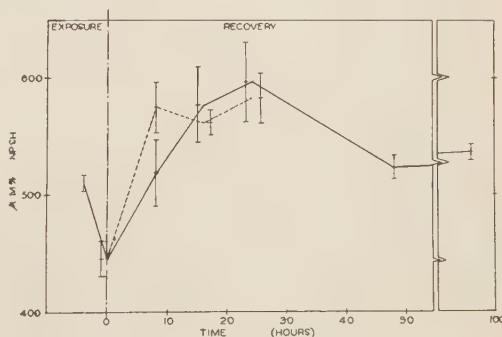


FIG. 2. Effect of fasting (hatched line) and non-fasting (solid line) on regeneration of kidney non-protein sulfhydryl compounds in rat after exposure to cold and restraint. Stand. error of mean indicated by vertical lines on curves.

mals, while Block(7,8) demonstrated that under normal conditions the *in vitro* rat liver produced 32-65 μ M % GSH per hour. The rate of recovery in the present experiments was somewhat slower than that which would be predicted from calculations based on these figures, even if it is assumed that recovery did not begin until normal body temperature had been obtained.

From a knowledge of the difference in the response of fasting and nonfasting animals, it could be reasoned that the available energy may play an important role in regeneration of NPSH. The stimulating effect of added succinate, fumarate, or malonate on the *in vitro* synthesis of GSH confirms the impression that the formation of the NPSH (GSH at least) is associated with energy yielding reactions (7). In these stressed animals the adenosine triphosphate (ATP) would very probably have been low, and low ATP has been shown to depress GSH production(7).

Summary. 1. Adult female Sprague-Dawley rats were exposed to cold and restraint to effect a lowering of concentration of the total nonprotein sulfhydryl compounds (NPSH) in the liver. At the termination of this exposure the animals were divided into 2 categories (fasting and nonfasting) and were permitted to recover under normal laboratory conditions. During the recovery period animals were sacri-

ficed at intervals to trace the rate of regeneration of the liver and kidney NPSH. 2. In neither the fasting nor the nonfasting categories was there a significant regeneration of the liver NPSH in 8 hours. In the nonfasting recovery was virtually complete in 16 hours and complete in 24 hours. In the fasting animals recovery was slower in beginning and was not completed by 24 hours. The rate of loss of NPSH from the liver was considerably greater, in these experiments, than the rate of recovery. 3. Exposure to cold and restraint appeared to lower the kidney NPSH. Recovery was completed in fasting and nonfasting animals in less than 8 hours, with a transient overcompensation in both categories.

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Free Amino Acids and Glutathione of Normal and Syphilitic Rabbit Testes.* (21248)

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The successful *in vitro* cultivation of pathogenic *Treponema pallidum* remains one of the major unsolved problems in syphilology. In seeking solutions to the problem the metabolic requirements of avirulent strains of spirochetes purported to be *T. pallidum* were inves-

tigated. A chemically defined medium for the cultured Reiter strain of *T. pallidum* was found(1) but the results have not been of value in the attempted cultivation of the pathogenic organism. Conditions favoring the *in vitro* survival of the pathogenic (usually Nichols) strain of *T. pallidum* led to the development of the treponemal immobilization test of Nelson and Mayer(2). None of the

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suggested media have resulted in significant *in vitro* multiplication of pathogenic *T. pallidum*. Work from this laboratory(3) has indicated that there may be an early disassociation of virulence and motility with the result that organisms may lose their virulence in a period of 2 to 4 days even though their *in vitro* motility persists as long as 10 to 14 days. In view of this, it seems worthwhile to seek other approaches to the problem.

Pathogenic *T. pallidum*, like many other microorganisms, demonstrates a preference for certain tissues. In the rabbit, regardless of the route of inoculation, a syphilitic orchitis occurs. Thus, these tissues are usually employed as the site of inoculation. It was felt advisable to begin a comparative study of the biochemistry of these tissues in the hope that comparisons between susceptible and non-susceptible tissues, and comparisons between infected and non-infected susceptible tissues might suggest factors necessary for the growth of the organisms. This paper is concerned with changes in the free amino acids, glutathione, and solid content of normal and syphilitic rabbit testes.

Experimental. Free amino acids of testes. Preparation of protein-free amino acid concentrates. The initial steps were performed in a cold room at 5°C. Fifty-two g of testes (from 6 to 15 rabbits) were homogenized in a pre-cooled Waring blender with 104 ml (2 vol.) of cold distilled water. Duplicate 3 ml samples were removed for the estimation of solid content as determined by weight after drying for 24 hours at 100°C. To the balance of the homogenate 560 ml of cold absolute ethanol were added and the mixture stirred for one minute, filtered at room temperature with re-filtration until the filtrate was clear. The precipitate was then washed with 50 ml of 80% ethanol. The combined filtrates were evaporated to dryness in vacuum at 30°C. The residue was extracted with successive 10, 5, and 3 ml portions of 10% isopropyl alcohol in water. The resultant suspension was then intermittently shaken for a 30-minute period with 6 g of the strongly acidic exchange resin, Amberlite IR-105 (H). The resin was allowed to settle, and the supernatant, containing a flocculent precipitate of proteins and

other large molecules was decanted and discarded. The Amberlite was then washed 10 times with successive 15-ml portions of distilled water. These supernatants were discarded. The amino acids were then recovered from the Amberlite resin by shaking the resin for 15 minutes with 5 ml of 5 N ammonium hydroxide. The supernatant and 5 successive washes with 10 ml of distilled water were filtered by gravity. The combined filtrates were then evaporated to dryness in vacuum at 30°C and the residue was redissolved in 2.5 ml of 10% isopropyl alcohol in water. Aliquots of this solution were employed for filter paper chromatography. These amino acid concentrates could be stored for long periods of time at 5°C. Some of the amino acids crystallized from solution during prolonged storage, but on warming of the tube they promptly redissolved. *Chromatography of free amino acids.* The 2-dimensional method of Levy and Chung(4) was employed with certain modifications. One or 2 μ l of the protein-free amino acid concentrate were applied at a corner 3 inches from each edge of a 22.5 x 18.5 inch sheet of Whatman No. 52 paper. The applications were slowly dried on the paper with a hair drier. The chromatograms were run at room temperature in a Chromatocab. A butanol-acetic acid-water (4:1:5) mixture was used for the development in the first dimension. The descending method was used. For the second dimension, the developing mixture consisted of 120 g of redistilled phenol, 120 g of redistilled m-cresol, 34 ml of 0.15 M pH 9.4 sodium borate buffer, and 50 mg of ascorbic acid in 2.5 ml of distilled water. The amino acid spots were identified with the ninhydrin reagent of Levy and Chung, dissolved in methanol rather than ethanol. The reagent was sprayed on the paper with a glass sprayer. A Welch Densichron was employed to estimate the density of the amino acid spots. The area under the resultant curve was a function of the concentration of the given amino acid, and this area could be used to estimate the quantity of amino acid present. In the calibration of the quantitative procedure, amino acids were run singly and in mixtures to obtain planimeter values of the density readings within the range

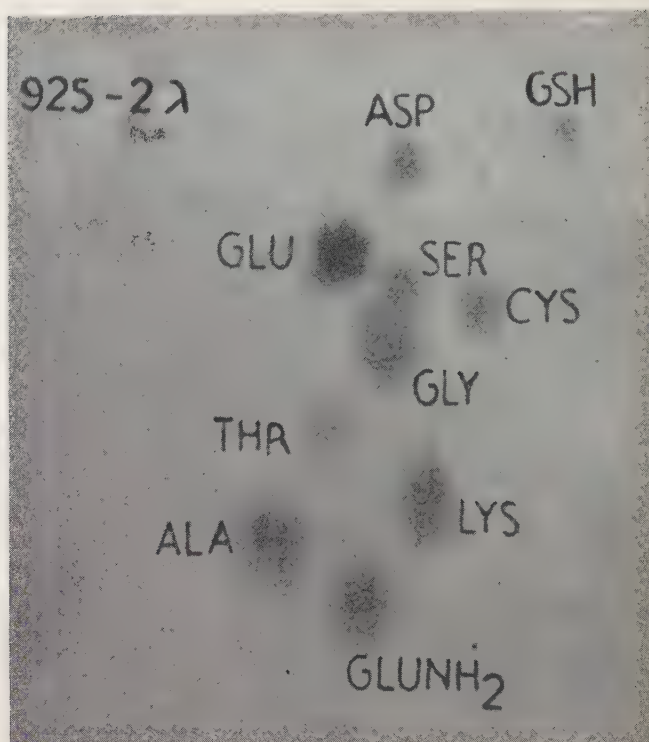


FIG. 1. Chromatogram of a protein-free Amberlite treated, amino acid concentrate of a syphilitic testes extract. The spots are those of 9 amino acids and of oxidized glutathione.

to be employed for quantitative work on the testes. A satisfactory calibration curve could not be obtained for the cystine standard and the values here reported are relative only and do not represent absolute values. These relative curves were based upon the densities of the alanine calibration curve. In early attempts to separate these amino acids we were unable to effect a separation of the serine, glycine, and cystine from the large quantities of reduced glutathione. After the Amberlite-ammonium adsorption and elution procedure was adopted no further difficulty was encountered since all of the glutathione was oxidized in the alkaline medium without being hydrolyzed. The R_f of the oxidized glutathione is extremely low and it does not interfere with the determination of the amino acids. Under the conditions of the present method nearly all of the amino acids gave a bluish purple color with ninhydrin. The glycine and the oxidized glutathione gave a reddish purple color and the asparagine, present only in trace amounts, gave a yellow color. The absolute

quantities of amino acids measured varied from 0.7 μg (glutamine) to 8.3 μg (glutamic acid). These represent the extremes in the 8 pools under study. *Reduced glutathione.* The reduced glutathione was determined in protein-free filtrates(5). A weighed quantity of freshly excised tissue (approximately 400 mg) was frozen in solid carbon dioxide and then homogenized in 3% metaphosphoric acid and one ml of cold distilled water. The Klett-Summerson colorimeter with green filter No. 54 was used to measure the intensity of the color produced by the addition of sodium nitroprusside. Within the range of our experiments (0.01-0.1 mg) the intensity of the color followed Beer's Law.

Results. Fig. 1 demonstrates a typical chromatogram obtained from a 2 μl sample of the amino acid concentrate from syphilitic testes. The location of aspartic acid, glutamic acid, serine, glycine, cystine, alanine, lysine, and glutamine are shown. In addition a trace of threonine is shown by the lighter spot. Not included in Fig. 1 are the more mobile

TABLE I. Free Amino Acid Content of Testes (mg/100 g on Dry Basis).

	Solids, %	Asp	Glu	Ser	Gly	Lys	GluNH ₂	Ala	Cys
Normal group									
	22.30	7.5	50.1	17.7	62.0	26.6	14.4	12.3	15.4
	24.15	5.4	65.8	9.9	52.6	45.0	21.6	16.9	15.8
	19.90	11.2	66.4	14.2	33.3	24.4	22.6	17.2	18.0
Syphilitic group									
(8)*	20.95	12.4	58.3	13.2	48.7	43.5	7.8	17.3	17.7
(13)	14.10	6.2	48.5	13.3	39.3	34.0	Trace	14.6	13.1
(27 to 82)	24.80	7.7	84.4	13.2	40.5	23.4	18.9	20.5	15.7
(80 to 104)	37.87	5.0	27.4	6.8	17.8	16.2	12.6	8.6	10.8
(156 to 180)	32.50	6.5	47.8	16.5	24.8	29.5	27.8	17.9	7.5

* Figures in parentheses indicate period of infection in days.

amino acids, methionine, leucine (or isoleucine), and asparagine (which gave a yellow color with ninhydrin) which are also present in trace amounts. The spot close to the point of application is that of oxidized glutathione. All of the glutathione is oxidized by the alkaline elution method. Adsorption on and subsequent elution from the strongly acidic Amberlite IR-105 (H) effected the removal of proteins and other large molecules and the partition of several amino acids after oxidation of the reduced glutathione.

In Table I is seen that during the most virulent stage of infection (8 to 13 days) there is a significant decrease in the free glutamine content of testes. There is a drop in the solid content of testes at 10 to 15 days of infection. The other free amino acid values, however, are not appreciably different from the normal values as observed during the experimental period of 8 days to 180 days of infection.

On a wet basis the reduced glutathione content of syphilitic rabbit testes average 65% of the normal value. On dry basis it is 74% of the normal value. The normal group's dry weight was 15.0-28.3%, the average being 21.9%. The average glutathione values, using 50 normal rabbits in 17 experiments, were found to be 95.1 mg on wet basis (highest 116, lowest 68) and 453.1 mg on dry basis (highest 713, lowest 263). In the syphilitic group (2-180 days incubation time), employing 45 rabbits in 14 experiments, the average was 62.0 mg on wet basis (highest 85, lowest

37), and 335.5 mg on dry basis (highest 509, lowest 126). All figures are per 100 g tissue. The dry weight was 10.8-38.6%, the average being 20.46%. The decreased reduced glutathione content of testes in syphilis is noteworthy. Recently Brückmann and Wertheimer(6) reported the following reduced glutathione values for normal rat organs: Pancreas, 60; kidney, 109; liver, 176 mg/100 g wet tissue. Our value of 95.1 mg (on wet basis) for normal rabbit testes is well within the range of those of rat kidney and rat liver.

Summary. A method for separating the free amino acids from proteins and other large molecules, and from interfering reduced glutathione has been devised. The strongly acidic exchange resin Amberlite IR-105 (H) has been employed for the adsorption of the free amino acids and ammonium hydroxide for their elution. The ammonia was removed *in vacuo*. The final purified concentrates were put in 10% isopropyl alcohol and subjected to 2-dimensional filter paper chromatography. The ninhydrin developed amino acid spots were measured by the densitometric method. The major amino acid components present in normal rabbit testes were aspartic acid, glutamic acid, serine, glycine, lysine, cystine, alanine, and glutamine. There were also present a trace of threonine, methionine, asparagine, and leucine (or isoleucine). In syphilis, during the most virulent stage of infection, there is a significant decrease in the free glutamine content of testes. Reduced glutathione, as determined by the quantitative nitroprusside test on protein free filtrates, was

found to be distinctly decreased in syphilitic testes.

The author wishes to thank Dr. Wilton E. Vanier for photographing the amino acid chromatogram, and Mr. John W. Clark, Jr. and Miss Martha Gaston for technical assistance.

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Effect of Aminopterin on Incorporation of C¹⁴ Formate into the 2 and 8 Carbons of Guanine.* (21249)

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Whether formate enters the 2 and 8 position of the purine ring(1) via the same immediate precursor or cofactor is not known. Its entry into the 2 position appears to require its prior incorporation into a reduced folic acid derivative in the presence of ATP (2). When aminopterin is injected into an animal this step may be partially blocked and it is possible that C¹⁴ formate incorporation might be so altered that the specific activity of the 2 carbon of purine would be different from that of the 8 carbon. This might occur if the immediate C¹⁴ precursors of the 2 and 8 carbons were different and the antimetabolite changed their pool sizes, or if a purine intermediate such as 5-amino-4-imidazolecarboxamide ribotide accumulated. However, in this study the C-2/C-8 ratio of C¹⁴ formate incorporation into rat nucleic acid guanine was consistently very close to 1 and did not change in the presence of aminopterin.

Methods. Guanine degraded in the following experiments was obtained from the nucleic acids of adult rats used in studies previously reported(3). All animals received C¹⁴ sodium formate,[†] 3.6 x 10⁷ cts/min/kg by intraperi-

toneal route, while some of the animals received simultaneously aminopterin, 50 mg/kg. All animals were sacrificed after 24 hours, and the sodium nucleate from different tissues obtained by hot 10% sodium chloride extraction and ethanol precipitation. In control experiments the following organs were employed: 1, total viscera; 2, total viscera plus added small intestine. In the aminopterin experiments total viscera plus intestine were used in No. 3, while small intestine was used in No. 4. 750 mg samples of sodium nucleate were refluxed in 50 ml of 0.5 N H₂SO₄ for 2 hours, filtered, and to the filtrate 50 ml of 10% AgNO₃ was added. The purine silver salt was washed, decomposed with HCl at 100° and treated with norite. After filtering, the volume was reduced to 7 ml and guanine crystallized out on neutralization. This was recrystallized from 2 N H₂SO₄. 10 mg of guanine sulfate in 1 ml of 4 N H₂SO₄ was converted to xanthine by addition of 30 mg of NaNO₂ in 0.7 ml of water at 75° over a 10-minute period. After cooling at 2°C overnight, the xanthine which crystallized was collected, and converted to uric acid by means of xanthine oxidase(4). The conversion was estimated spectrophotometrically(5). 84 mg of carrier uric acid was added as the lithium salt and uric acid was precipitated on acidification. This was repeated, and the resulting

* The radioactive isotope used in these studies was obtained on allocation from the Atomic Energy Commission.

† Sodium formate 0.28 mM/mc. dissolved in 0.775 ml of dilute alkali.

TABLE I. Effect of Aminopterin on Incorporation of C^{14} Formate into the 2 and 8 Positions of the Guanine. Four experiments. Cts/min./mM carbon.

Control		Ratio 2/8	Aminopterin		Ratio 2/8
C-2	C-8		C-2	C-8	
5140	5260	.98	5400	5640	.96
31400	32500	.97	19200	19400	.99

precipitate extracted twice with hot ethanol and dried with ether. The degradation of the uric acid described in detail elsewhere(1,6), involved a perchlorate oxidation of the purine ring to urea (C-8) and alloxan; the latter was reduced with H_2S to alloxantin and this then oxidized with PbO_2 to urea (C-2). Both samples of urea were degraded with urease and the CO_2 evolved was trapped in NaOH and plated as $BaCO_3$. Samples were counted with an end window Geiger-Muller tube to a statistical error of 2%.

Results. Because samples taken for different experiments (Table I) were not comparable, no conclusions can be drawn from this data regarding the effect of aminopterin on the total C^{14} formate incorporation into guanine. An extensive study of this effect has been reported elsewhere(3). However, the results do indicate that under these conditions, aminopterin has no effect on the ratio of incorporation of C^{14} -formate into the 2 and 8 positions of guanine. This confirms the conclusions of Drysdale *et al.*(6) drawn from data which showed some variation in the ratio of C-2 to C-8 activity. It is impossible to conclude from the present study that the formate derivative which is the precursor for

C-2 is the same as that for C-8. However, if the precursors differ, they must have attained the same specific activity from C^{14} -formate, not only in the different tissue samples but also in the presence or absence of aminopterin.

Further evidence to support the concept that the incorporation of formate into the 2 and 8 carbons is via the same pathway has been obtained recently by *in vitro* experiments on the synthesis of a formylated glycine amide ribotide(7). The cofactor present in boiled extract which is necessary for the synthesis of this compound can be replaced by citrovorum factor(8), under conditions comparable to those required for the introduction of formate into the 2 position(2).

Summary. The ratios of specific activity of carbon 2 vs. carbon 8 of rat nucleic acid guanine, labeled *in vivo* with C^{14} formate, remains constant in aminopterin treated animals compared to controls.

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Influence of Hyperglycemic Factor (Glucagon) on 17-Hydroxysteroids in Plasma and Urine of Man. (21250)

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Abundant evidence indicates that hyperglycemic factor exerts its effect by stimulating hepatic glycogenolysis. Sutherland(1) has demonstrated that hyperglycemic factor accelerates resynthesis of active hepatic phosphorylase, the phosphorylase reaction being the limiting one in the enzymatic transformation of glycogen to glucose. Another method by which hyperglycemia may occur involves the accentuation of gluconeogenesis, and perhaps inhibition of glucose utilization, via adrenal stimulation(2). That this may be a minor, though significant, effect of glucagon has not been adequately investigated. Kirtley *et al.*(3) found an increase in urinary 17-hydroxycorticoid/creatinine excretion in 3 normal and 9 diabetic patients following the administration of hyperglycemic factor, and postulated the presence of adrenal stimulation, perhaps secondary to the stress of administration rather than to an effect of hyperglycemic factor *per se*. The observations of Vuylsteke *et al.*(4) that hyperglycemic factor caused a decrease in eosinophils in the dog, also raised the possibility of adrenal stimulation.

It was our purpose to investigate several non-diabetic persons in an attempt to reproduce the findings of Kirtley's group, and in addition, to observe whether rises occurred in plasma 17-hydroxysteroid levels.

Method. Eight hospital patients with normal renal and hepatic function and no signs of congestive heart failure were chosen for study. These patients were fasted for 14 hours prior to onset of the experiment. In 4 patients, purified hyperglycemic factor† was given by intravenous infusion over a 30-min. period in a dose of 10 µg/kg. Blood glucose

and plasma 17-hydroxysteroid determinations were performed on samples taken just before and at the end of infusion, and another 17-hydroxysteroid determination on plasma was made at the end of one hour following the start of infusion. The plasma 17-hydroxysteroid determinations were performed according to the method of Nelson and Samuels(5).‡ A 3-hour control urine specimen was collected prior to infusion, and 2-hour specimens were collected during and after the administration of hyperglycemic factor. Urinary 17-hydroxysteroid determinations were performed by the method of Glenn and Nelson(6). In the remaining 4 patients, hyperglycemic factor was given in similar doses, but injected intravenously over a 30-second period. Blood sugar responses were again noted, and plasma 17-hydroxysteroid determinations performed before, at 20 and 40 minutes and, in 2 cases, 80 minutes, following injection. Urinary steroids were performed on 3-hour, instead of

TABLE I. Effect of 30-Minute Infusion of Hyperglycemic Factor (Lilly Lot 208-158B-214) on 17-hydroxysteroid Levels on 4 Patients.

Age	Min.	Blood sugar, mg/100 cc	Min.	17-hydroxysteroids	
				Plasma, µg/100 cc	Urine, µg/hr
15	0	98	0	8	B*
	30	165	30	4	0-2 hr 325
			60	4	2-4 135
16			0	8	B
			30	8	0-2 hr 140
			60	7	2-4 50
75	0	91	0	7	B
	30	165	30	8	0-2 hr 205
			60	5	2-4 205
67	0	110	0	15	B
	30	152	30	8	0-2 hr <50
			60	8	2-4 469

* B = Baseline.

‡ The authors wish to express their appreciation to Dr. Kris Eik-Nes for his valuable personal advice concerning certain details of this method.

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TABLE II. Effect of 30-Second Injection of Hyperglycemic Factor (Lilly Lot 208-158B-34) on 17-hydroxysteroid Levels on 4 Patients.

Age	Min.	Blood sugar, mg/100 cc	17-hydroxysteroids		
			Min.	Plasma, μg/100 cc	Urine, μg/hr
60	0	110	0	13	
	30	157	20	12	—
			40	16	
49			0	11	
			20	11	—
			40	8	
80			0	14	B* 181
			20	13	0-3 hr 75
			40	13	3-6 146
			80	14	
49			0	0	B 108
			20	<2	0-3 hr 80
			40	0	3-6 70
			80	<2	

* B = Baseline.

2-hour, specimens, and on only 2 patients.

Results. Table I and II show the data obtained in the 2 groups of patients. We did not observe a significant rise in urinary steroid levels in those patients who received a rapid injection of hyperglycemic factor (Table II). In those receiving the intravenous infusion, urinary excretion of steroids rose significantly in patients 1 and 3. The low value for the 0-2-hour urine specimen of patient 4 is hard to understand; no difficulty in collecting the sample was experienced. The moderately elevated 2-4-hour specimen does not necessarily imply an elevated 0-2-hour sample, so no conclusions can be reached concerning the values for this patient. In none of the patients was a significant elevation of plasma 17-hydroxysteroids observed. The values for plasma steroids compared well with the normal values reported by Bliss *et al.* (7).

Discussion. The lack of rise of plasma steroid in response to hyperglycemic factor makes it very unlikely that any of the metabolic effects of this material can be attributed to adrenal gland stimulation. Although the recent studies of Bongiovanni (8) have shown a much larger yield of 17-hydroxysteroids from plasma after glucuronidase hydrolysis, there is as yet no evidence that adrenal stimulation can produce a rise in conjugated steroids in the absence of an elevation in unbound steroids.

In those 4 patients who received a 30-minute infusion of hyperglycemic factor, an increase in urinary excretion of steroids occurred in 2. These changes were similar to those reported by Kirtley *et al.* (3). These observations, being variable from patient to patient, and not apparent in the group subjected to rapid injection, support the proposal that the increased steroid excretion was a response to stress (3). The variability in our findings also makes it improbable that the hyperglycemic factor has any action on the renal mechanism responsible for steroid excretion. In the patients who showed a rise in urinary steroid excretion, apparently due to stress, one might question why the plasma levels did not also rise. Some elevation in these levels might be expected during the second hour but unfortunately we did not measure plasma levels in this period to clarify this point. However, a response occurring this long after the administration of hyperglycemic factor should have little relation to its metabolic effect, which is almost entirely dissipated within one-half hour after administration (3,9).

The alternate postulation raised by Vuylsteke's group, that the eosinophil drop after hyperglycemic factor is independent of the adrenal cortex, and thus similar to that produced by epinephrine or intravenous glucose, is also more compatible with our observations.

Summary. 1. Plasma 17-hydroxysteroid levels and urinary 17-hydroxysteroid excretion rates were observed in humans before, during, and after the intravenous administration of purified hyperglycemic factor. There were no significant changes in plasma values, but some patients showed elevation in urinary excretion of steroids, apparently related to the degree of stress incurred. 2. By these methods, we could observe no direct action of hyperglycemic factor on the adrenal cortex.

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Chelating Agents, Barbiturates, and Stimulation of Rat Brain Adenosinetriphosphatase. (21251)

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Recent studies have indicated that barbiturates may, *in vitro*, interfere with oxidative phosphorylation(1). The significance of this interference is difficult to assess, since many compounds of apparently unrelated biological activity and structure exhibit the same effect. However, further understanding of the manner in which oxidative phosphorylation is interrupted may reveal points of departure among the various compounds involved. In a previous discussion(2), it was pointed out that certain broad-spectrum antibiotics, as well as 2,4-dinitrophenol(3), increased the adenosinetriphosphatase (ATPase)* activity of rat liver homogenates and mitochondria. It was also observed(2) that the ATPase activity of rat brain homogenates was not stimulated by either the antibiotics or dinitrophenol. The results obtained in the present study indicate a possible role of chelation effects in ATPase stimulation.

Materials and methods. The purities of Sigma sodium adenosinetriphosphate (ATP), Sigma sodium adenosinediphosphate (ADP), and Pabst sodium uridinetriphosphate (UTP) were confirmed by analyses of labile and total phosphorus. The barbiturates† and other chemicals used were the purest obtainable

from various commercial sources. Ten per cent homogenates of rat liver or brain (frontal lobes) were made in 0.25 M sucrose at 0°C by use of a glass homogenizer(5). Tubes were set up containing the following: 0.04 M KCl, 0.0001 M MgSO₄, 0.05 M tris(hydroxymethyl)amino-methane buffer (pH 7.4), 0.003 M ATP (or other substrate), 0.1 ml homogenate, barbiturate solution or other additions if desired, total volume 1.0 ml. All solutions were made in glass-redistilled water and adjusted to pH 7.4. Substrate was added after the other components of the reaction mixture had been equilibrated in the water bath for 5 minutes. Following 10 minute incubation at 30°C, proteins were precipitated by addition of 0.1 ml 50% trichloroacetic acid. Orthophosphate was determined(6) in the supernatant after centrifugation. Corrections were made for phosphorus liberation in the absence of homogenate and in the absence of substrate. The level of magnesium ion used in these experiments was purposely set far below the optimum in order to allow for maximum expression of stimulatory and chelation effects.

Results. In these experiments, the reproducibility of results when duplicate determinations were made with the same homogenate was quite satisfactory, a variation of more than $\pm 5\%$ being unusual. However, the activities and stimulatory responses of tissues from different animals were often subject to considerable quantitative variation. For this reason, comparisons of stimulatory activity

* This term is used to identify enzyme(s) catalyzing release of inorganic phosphate from ATP. The exact mechanism involved may be more complex than that of a direct hydrolysis of substrate(4).

† The authors are indebted to Dr. C. W. Pettinga, Eli Lilly and Co., for a sample of 1,3-dimethyl-butyl-ethyl-barbiturate.

TABLE I. Barbiturate Stimulation of Rat Liver ATPase.

Barbiturate (1×10^{-3} M)	% increase in activity, avg (range)
Barbital	15 (8- 22)
Phenobarbital	31 (23- 33)
Pentobarbital	44 (33- 59)
Thiamylal	90 (77-100)
Thiopental	155 (116-195)

among various compounds are drawn from experiments in which all of the compounds were tested with the same homogenate, and the results are most conveniently expressed as the per cent increase in phosphorus liberated over the control homogenate. The average values and ranges given represent experiments with at least 3 animals for each compound listed.

Barbiturate stimulation of liver ATPase. As shown in Table I, there were definite variations in potency among the barbiturates tested. While this observation was of interest in itself, it was felt that it would be more appropriate to examine effects of barbiturates on brain tissue reactions. However, it was not possible to influence the ATPase activity of brain homogenates by addition of high levels of barbiturates, chloramphenicol, chlortetracycline, or 2,4-dinitrophenol. The same negative results were obtained with homogenates prepared from intestinal muscle, spleen, and bone marrow.

Stimulation of brain ATPase. The report of Chappell and Perry (7) that pigeon muscle mitochondria isolated in the presence of ethylenediamine-tetraacetic acid (EDTA) exhibited a lowered ATPase activity which could be stimulated by dinitrophenol led to the trial of this technic with brain homogenates. The results, summarized in Table II, clearly indicated that homogenization of the brain in the presence of 1×10^{-3} M EDTA resulted in a lower ATPase level which could then be increased by stimulating agents which had been found effective with liver ATPase. The one exception among the compounds tested was oxytetracycline, which was apparently still inert in the brain system. It was also determined that it was not necessary to add the EDTA previous to homogenization. Similar results were obtained when 1×10^{-4} M EDTA

was added after homogenization in sucrose. This latter procedure was followed as a matter of convenience in many subsequent experiments.

Effect of other chelating agents. The following compounds had little or no effect on the initial brain ATPase level, nor did their presence bring about significant stimulation by 1×10^{-3} M pentobarbital, 1×10^{-4} M dinitrophenol, or 1×10^{-4} M chlortetracycline: 1×10^{-3} M citric acid, carboxymethylmercaptosuccinic acid, acetylacetone, ammoniadiaacetic acid, N,N-bis(2-hydroxyethyl)glycine, N,N-bis(carboxymethylethanolamine, and 8-hydroxyquinoline. Ammoniatricacetic acid, 1×10^{-3} M, and 1,2-diaminocyclohexane-N,N'-tetraacetic acid, 1×10^{-4} M, gave results which were comparable to those obtained with EDTA.

Other substrates. Under the conditions used in these experiments with brain homogenates, there was no measurable production of orthophosphate from pyrophosphate or adenylic acid. ADP was degraded about half as rapidly as ATP, and stimulatory effects of dinitrophenol, chlortetracycline, and pentobarbital were of the same order of magnitude (Table III). UTP degradation by liver homogenates was exceedingly sensitive to all these stimulators, while only chlortetracycline produced a marked increase in the activity of the brain-EDTA homogenates.

Discussion. The 3 chelating agents which were effective in reducing ATPase activity and allowing subsequent stimulation are not-

TABLE II. Stimulation of Rat Brain ATPase.*

Agent	Concentration (M)	% increase in activity, avg (range)
Barbital	1×10^{-3}	15 (7- 20)
Phenobarbital	"	26 (25- 27)
Thiamylal	"	50 (40- 60)
Amytal	"	76 (62- 93)
Pentobarbital	"	91 (86-100)
1,3-Dimethyl-butyl-ethyl barbiturate	"	79 (73- 86)
2,4-Dinitrophenol	1×10^{-4}	93 (67-133)
Chlortetracycline	"	104 (75-133)
Chloramphenicol	1.55×10^{-3}	59 (58- 60)

* Homogenates prepared in presence of 1×10^{-3} M EDTA. Control homogenates produced avg of 15γ P (13-19) as compared to 33γ P (28-43) for homogenates prepared in absence of EDTA.

TABLE III. Stimulation of Adenosinediphosphate and Uridinetriphosphate Degradation in Rat Brain and Liver Homogenates.

Substrate	Tissue*	Stimulator (M)	γ P Liberated Stimulator	
			Absent	Present
Adenosinediphosphate	Brain	2,4-Dinitrophenol, 1×10^{-4}	8	15
		Chlortetracycline, "	8	18
		Pentobarbital, 1×10^{-3}	8	14
Uridinetriphosphate	"	2,4-Dinitrophenol, 1×10^{-4}	9	10
		Chlortetracycline, "	9	18
		Pentobarbital, 1×10^{-3}	9	11
	Liver	2,4-Dinitrophenol, 1×10^{-4}	6	41
		Chlortetracycline, "	6	25
		Pentobarbital, 5×10^{-4}	6	15

* Brain homogenates made in presence of 10^{-3} M EDTA; liver homogenates in 0.25 M sucrose only.

able for their enormous affinity for magnesium ion: $\log K^a$ values(8) are 7.0 for ammonia-triacetic acid, 8.69 for EDTA, and 10.3 for 1,2-diaminocyclohexane-N,N'-tetraacetic acid. The possibility was considered that the stimulating agents might act by a modification of chelate stability. No indication for such an effect of chloramphenicol or 2,4-dinitrophenol on the EDTA-Mg⁺⁺ chelate could be seen in titration studies patterned after those of Schwarzenbach and Ackermann(9), nor could any evidence be obtained in this way for the formation of Mg⁺⁺ complexes by the various stimulating agents employed. Obviously such negative results are inconclusive and a different approach to the problem may be more rewarding.

Speculation as to the possible significance of ATPase stimulation by barbiturates is restrained by the finding that the convulsive compound(10), 1,3-dimethyl-butyl-ethyl-barbiturate, was also active in this respect, as it is in the uncoupling of oxidation and phosphorylation(1). The greatest value to be derived from these observations at the present time is an awareness of the fact that various compounds may stimulate the breakdown of ATP (and ADP and UTP) under certain conditions and by so doing may influence the course of other reactions dependent on a supply or balance of high-energy phosphate compounds.

Summary. 1. The ATPase activity of rat brain homogenates prepared in 0.25 M sucrose

was not affected by 1×10^{-4} M 2,4-dinitrophenol, 1×10^{-4} M chlortetracycline, 1.55×10^{-3} M chloramphenicol, or 1×10^{-3} M levels of various barbiturates, in contrast to the effects of these compounds on liver homogenates prepared in the same way. 2. If EDTA was added either before or after homogenization, so that the final concentration in the reaction mixture was 1×10^{-4} M, the ATPase activity of the brain homogenates was considerably reduced and susceptible to stimulation by the above compounds.

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Relation of Survival Time to Implantation Time of Second Set Skin Homografts in the Rat.* (21252)

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It is fairly well established that a second homograft of skin or other organs from the same donor will be rejected more rapidly than the first. Kidneys transplanted to guinea pigs previously immunized with emulsions of guinea pig kidney were rejected sooner than kidneys put into non-immune animals(1). Skin homografts in several human burn cases sensitized the patient to the extent that a subsequent homograft from the same donor caused an anaphylactic type reaction(2). More recently, it has been shown that second set skin homografts in human beings persist for a shorter time than do the first set grafts (3-5). An exception has been noted in a human case where the second set graft lasted nearly twice as long as the first set(12). Work on the rabbit(6-9), the guinea pig(10), and the dog(11) has demonstrated that a second skin homograft from the same donor does not persist as long as does the first. The so-called second set phenomenon is very strong evidence in favor of Medawar's theory of acquired immunity(6,7). However, several points remain to be cleared up. Medawar(6) and Lehrfeld and Taylor(13) showed that a first set skin homograft induces a systemic response which causes destruction of the graft. This response, induced by the first set graft, apparently destroys any ensuing grafts from the same donor. When this response first appears, when it reaches its peak and when it is no longer present are questions which need answering. This study proposes to answer these questions by implanting second set grafts while the first sets are still surviving, at the time of their rejection and at various times after rejection. Furthermore, very little work has been done on third set homografts. Studies on third, fourth and fifth set homografts used in the treatment of burn cases have shown that

each successive graft has a survival time shorter than the one before(5). Third set corneal grafts onto the thoracic wall of rabbits have been shown to survive for the same length of time as do second set grafts(14).

Material and methods. All animals used were male albino rats, 6-8 weeks old, of the Sherman and Wistar strains. They were obtained from 2 different dealers in order to avoid the possibility of genetic similarity between the 2 animals of each homografted pair. The grafting procedure employed has been given in detail by Lehrfeld and Taylor (13). All animals were conditioned by a large first set homograft sewed into the upper dorsum. These grafts ranged in area from 100-225 sq mm and were removed at the level of, but not including the panniculus carnosus. In all cases, the same donor supplied the first and second set grafts. The second set homografts were added to the mid-dorsal region of the host simultaneously with the first set graft, or 5, 6, 7, 8, 9, 11, 12, 15, 18, 30 and 60 days after implantation of the first set graft. These second set grafts were either large grafts (100-225 sq mm) or pinch grafts (1-4 sq mm). In addition, third set grafts were placed on a number of animals after rejection of the second set grafts. Dressing changes and observations were performed daily with the animal under ether anesthesia, according to the technic of Taylor and Lehrfeld(15). Observations of the grafts were done through mineral oil under a stereoscopic dissecting microscope at 90 x magnification. The homograft was considered rejected at the time that hemal stasis was observed in the vascular system of the graft (15).

Results. The results of the experiment are illustrated in Table I. The mean survival time of the conditioning grafts was 8.14 days. Pinch grafts, when added simultaneously with the first set grafts, survived for 8.1 days. Second set grafts added 5 days after the first

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TABLE I. Comparison of Survival Times of 1st and 2nd Set Skin Homografts.

No. of cases	Mean 1st set survival time, days	Interval between 1st & 2nd set graft, days	Type of 2nd set graft	Mean 2nd set survival time, days
10	8.1	0	P*	8.1
8	8.5	5	L (4)	3.0
	7.25	5	P (4)	2.25
12	7.75	6	L (6)	0
	8.0	6	P (6)	0
10	8.3	7	L (6)	0
	8.0	7	P (4)	0
6	8.2	8	L	5.0
2	8.5	9	L	5.0
14	8.3	11	L (10)	5.3
	8.25	11	P (4)	5.0
6	8.1	12	L	4.7
4	8.25	15	L	5.5
2	8.75	18	P	5.0
8	8.5	30	L	5.5
8	8.5	60	L	8.0

* P = Pinch; L = Large.

set implantation survived for 2.62 days. Second set grafts added 6 and 7 days after implantation of the first set never became revascularized and are considered to have a survival time of zero days. Grafts added 8, 9, 11, 12, 15, 18 and 30 days after implantation of the first set graft showed a mean survival time of 5.1 days and in no case did any of these grafts survive longer than 6 days or less than 4 days.

The survival time of second set grafts implanted 60 days after implantation of the first set graft was 8 days, which is a typical first set survival time, showing no accelerated breakdown.

In 6 rats, third set homografts were implanted 7 days after implantation of the second set graft. These survived 4.8 days as compared to 4.7 days for the second set graft.

Discussion. Two facts emerge from these results: 1. The survival time of second set skin homografts is definitely and consistently shorter than that of the first set graft. 2. The factor determining the length of survival time of second set homografts is the time relationship between implantation of first and second set grafts. Second set grafts implanted simul-

taneously with, or up to 5 days after implantation of the first or conditioning grafts, become revascularized in the same way as do the first set grafts. The rejection of these grafts is identical to, and occurs simultaneously with, the rejection of the first set grafts.

Second set grafts implanted on the 6th and 7th days after implanting the first set, a time at which the first set graft is still surviving, never became revascularized and appear to have a survival time of zero days. This occurs even with very small pinch grafts which are capable of becoming revascularized within 24 hours(13). Second set grafts implanted from 8-30 days after implantation of the first set graft survive for a mean time of 5.1 days, while those implanted 60 days after the first set, had returned to a normal first set survival time of 8 days. There was no significant difference between the survival time of second and third set grafts when the third set graft was implanted 7 days after the second.

The systemic response invoked by the conditioning graft apparently functions in the destruction of any second set graft implanted before the rejection of the first set graft. This response appears to reach maximum effectiveness at the 6th and 7th days as shown by the fact that second set grafts implanted during this time do not become revascularized, although if implanted 24 hours earlier or later they develop an active hemal circulation before their rejection.

The 5-day survival time exhibited by second set grafts implanted between 8 and 30 days after the first set grafting indicates that the systemic response elicited by the first graft has diminished in intensity to a point where it cannot destroy the second graft immediately. The temporal sequence in the accelerated breakdown of these second set grafts is suggestive of an anamnestic type response by which a mechanism, having already been primed by contact with the first set graft, becomes effective enough to destroy the second set graft in only 5 days. Somewhere between 30 and 60 days, this type of response is exhausted and second set homografts implanted at this time survive for 8 days, which is normal first set survival time.

Summary and conclusion. 1. Relation of

survival time to implantation time of second set skin homografts in the rat was studied. 2. Second set homografts implanted simultaneously with, or 5 days after the first set graft, develop an active hemal circulation and are rejected simultaneously with first set graft. 3. Second set homografts implanted 6 or 7 days after first set graft never become revascularized and are considered to survive for zero days. 4. Second set homografts implanted 8-30 days after first set graft develop an active hemal circulation and survive for a mean time of 5.1 days. 5. Second set homografts implanted 60 days after first set graft show a normal first set survival time of 8 days. 6. There is no significant difference in survival time of second and third set grafts when third set is implanted 7 days after the second set graft. 7. It is concluded that temporal sequence in accelerated breakdown of second set skin homografts is suggestive of an immunological mechanism triggered by the host's contact with the first set graft. This is in agreement with Medawar's theory of acquired immunity.

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Biosynthesis of Ascorbic Acid and Prevention of Glycogen Depletion in Liver and Muscle. (21253)

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We previously reported that gradual accumulation of fat metabolites (like acetoacetate and β -hydroxybutyrate in the systems of rabbits and guinea pigs) is responsible not only for the onset of hyperglycemia following a hypoglycemia but also for causing decreased glucose tolerance, depletion of reduced glutathione of blood and other diabetic symptoms (1-6). Tidwell and Nagler(7) observed that fasting blood sugar level of rats were significantly depressed by repeated daily injections of acetoacetate, without showing its eventual rise as in rabbits. They referred to the work of Parnes and Wertheimer(8) to explain such effect on blood sugar levels of rats injected with acetoacetate on the basis of decreased

glycogenolysis or increased glycogenesis. We have failed to corroborate such observations. They reported, on the other hand, that acetoacetate markedly increased glycogenolysis(9). That acetoacetate depresses glycogenesis is also evident from the observation of Chari and Wertheimer(10) that acetoacetate has a specific effect on glycogen synthesis. Tidwell and Nagler(7) also referred to our work(11) stating that acetoacetate markedly depletes glycogen storage in liver and muscle of rats. This statement also seems inaccurate. Most of our work was done on rabbits. We never reported that sodium acetoacetate depletes liver and muscle glycogen of rats. It is likely that species difference might play an import-

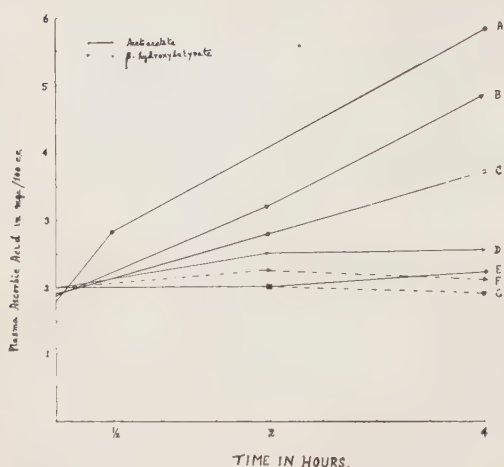


FIG. 1. Biosynthesis of ascorbic acid in rats after acetoacetate and β -hydroxybutyrate injections.

Doses in mg/kg body wt:

A 180	C 300	E 600	G 500
B 200	D 500	F 50	

ant part in response to diabetogenic substances.

Ascorbic acid is highly beneficial in improving glycogen synthesis(12) as well as ketolysis(13). According to Mosonyi(14) animals which can synthesize ascorbic acid react to administration of chemicals, especially some ketones by increasing their ascorbic acid excretion. Attempts, therefore, were made to see if such difference in behavior between rats and rabbits induced by acetoacetate could be accounted for through difference in capacity for immediate biosynthesis of ascorbic acid caused through injection of "acetone bodies" such as acetoacetate and β -hydroxybutyrate.

Experimental. Twenty-four healthy albino rats each weighing approximately 100-150 g and 16 rabbits each weighing approximately 2 kg were selected. Rats were maintained on the laboratory stock diet. Rabbits were given soaked Bengal gram (*Cicer arietinum*). Sodium acetoacetate and β -hydroxybutyrate were injected intramuscularly in doses shown in Fig. 1. Blood was collected from the marginal ear vein of rabbits. As the rats did not give sufficient blood for ascorbic acid estimation, equal quantities of blood from tails of 2 animals were mixed. Ascorbic acid was determined colorimetrically with indophenol dye according to the method of Bessey(15). Re-

sults are shown graphically in Fig. 1. Results with rabbits are represented in Fig. 2.

Since acetoacetate in higher concentration depresses biosynthesis of ascorbic acid in rats (Fig. 1), we investigated whether such high doses of acetoacetate would deplete glycogen storage of their liver and muscle. Twenty-six young rats weighing 100-150 g were fed the same diet as in the previous experiment(11). Ten animals were given daily injection of 500 mg/kg body weight of sodium acetoacetate, and 8 animals given dose of 200 mg/kg body weight. The animals were killed at intervals and glycogen in liver and muscle estimated according to the method of Good, Krammer, and Somogyi(16). Blood sugar was estimated according to the method of Hagedorn and Jansen(17). The results are shown in Table I.

In order to see the cumulative effect of sodium acetoacetate and β -hydroxybutyrate on blood ascorbic acid, 4 rats were injected daily with 200 mg/kg body weight of acetoacetate and 4 rats with β -hydroxybutyrate in daily dose of 50 mg/kg body weight. Animals were killed by decapitation after 55 days injection and plasma ascorbic acid estimated according to the method of Bessey(15). Results are shown in Table II.

Results. The results recorded in Figs. 1 and 2 illustrate that although rabbits are unable to synthesize ascorbic acid when injected

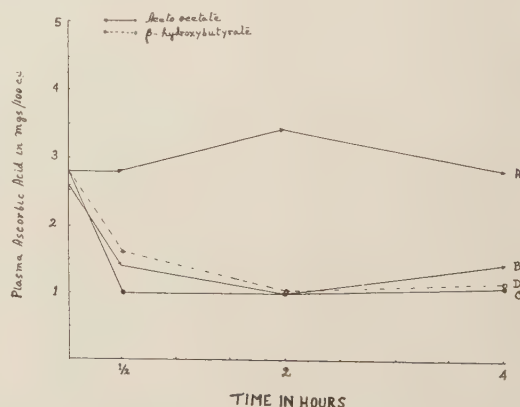


FIG. 2. Biosynthesis of ascorbic acid in rabbits after acetoacetate and β -hydroxybutyrate injections. Doses in mg/kg body wt:

A 60	B 180	C 500	D 500
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TABLE I. Effect of Sodium Acetoacetate on Blood Sugar and Glycogen Level of Liver and Muscle in Rats. (Animals were killed after 18 hr fasting.)

No. of animals	Dose of acetoacetate	Period of inj. (days)	Glycogen, mg/100 g tissue		Blood sugar in mg/100 cc
			Liver	Muscle	
4	0	0	260 \pm 8.2	128 \pm 10.2	82 \pm 1.5
2	200	10	250 \pm 10.2	140 \pm 6.1	78 \pm 2.5
2		25	255 \pm 8.8	128 \pm 8.2	70 \pm 3.8
2		40	248 \pm 5.8	120 \pm 10.2	72 \pm 4.2
2		55	240 \pm 6.2	130 \pm 10.8	72 \pm 4.3
2	500	10	202.2 \pm 15.2	160 \pm 12.2	65.0 \pm 1.2
3		30	140.6 \pm 7.8	47.3 \pm 5.8	140.0 \pm 8.2
3		55	88.6 \pm 6.5	25.3 \pm 3.2	190.0 \pm 10.2
2*		69	—	—	210 \pm 8.2

* The animals showed glycosuria (.36%) on 70th day.

TABLE II. Effect of Repeated Daily Injection of Acetoacetate and β -hydroxybutyrate (Sodium Salt) on Plasma Ascorbic Acid.

No. of animals	Substance injected	Dose, mg/kg	Period of inj. (days)	Plasma ascorbic acid, mg/100 cc
4	0	0	0	2.08 \pm .12
4	Acetoacetate	200	55	1.98 \pm .20
3		500	55	.88 \pm .02
4	β -hydroxybutyrate	50	55	.98 \pm .04

with acetoacetate, beyond a certain concentration, rats are capable of doing so depending inversely on concentration of acetoacetate. With concentrations as high as 600 mg/kg there was a definite depression in the process. β -hydroxybutyrate showed, however, no effect on biosynthesis of ascorbic acid either in rats or rabbits.

It is also evident (Table I) that acetoacetate in very high concentration can deplete liver and muscle glycogen even in rats and can bring about a marked increase in blood sugar concentration on repeated injection for a number of weeks. Tidwell and Nagler (18) began with a comparatively low concentration of acetoacetate and could not observe any such effect. Our work with lower concentration of acetoacetate also shows similar result (Table I). The failure of acetoacetate in depleting liver and muscle glycogen of rats when injected in such low concentration may be attributed to the biosynthesis of ascorbic acid which has been found to be stimulated by acetoacetate up to a certain concentration.

Summary. 1. Injection of sodium acetoacetate, in low concentrations (up to 200 mg/kg) stimulated biosynthesis of ascorbic acid in rats and did not disturb carbohydrate metabolism by raising blood sugar level. 2.

This compound at higher concentration (600 mg/kg), however, depressed such biosynthesis, raised blood sugar and depleted liver and muscle glycogen in rats. 3. Sodium β -hydroxybutyrate depressed biosynthesis of ascorbic acid in rats. 4. Rabbits failed to synthesize ascorbic acid after acetoacetate or β -hydroxybutyrate injection. 5. Depression in biosynthesis of ascorbic acid in animals is shown to be associated with disturbance in carbohydrate metabolism.

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Demonstration of Anatomy of the Giant Fiber System of the Squid by Microinjection.* (21254)

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There are a number of morphological, physiological and pharmacological technics available for the study of the gross and microscopical features of the nervous system. In this paper is described still another technic, that of microinjection which has been adapted to demonstrate the finer structures at the cellular and intercellular levels. With this technic, single neurons can be colored and their exact courses, branches and connection traced.

Materials and methods. The squid (*Loligo pealii*) was used, the gross aspects of the giant fiber system having been demonstrated by Young(1). If only the mantle fibers and the stellate ganglion were needed, the animal was decapitated; if the central nervous system was needed intact, the animals were narcotized with chloral hydrate (1 g/liter filtered sea water). The mantle was opened by a mid-ventral incision, flattened against a glass plate, and firmly anchored (Fig. 1). The specimen was kept wet with sea water during its use. It was not necessary to remove the fibers from the mantle because of the latter's translucence. However, when the second order fiber from the stellate ganglion into the central nervous system was to be injected, it had to be dissected free from the other fibers making up the bundle. A binocular dissecting microscope was adequate. The specimen on a glass plate was lighted from below. A simple right-angle coarse-gearied micromanipulator was used.

Micropipettes with tips 5-10 μ were hand drawn from 0.85 mm bore hard glass tubing, according to the method of Barber as modified and extensively used by Chambers(2,3). Pipettes were filled just before use with 0.1-0.5% Trypan Blue or 1% Trypan Red(4). Under the dissecting microscope the microtip

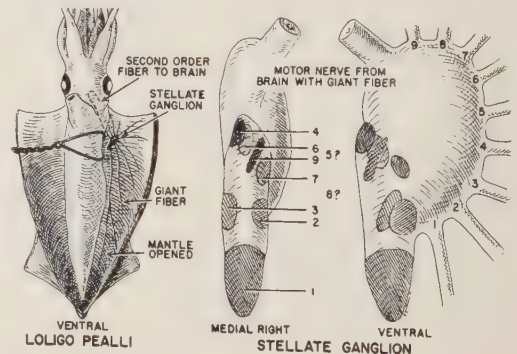


FIG. 1. The giant fibers of the mantle decrease in size from posterior to anterior so that fiber 1 is huge compared with 8 or 9. Nucleus 1—Several injections. This is the largest cell mass because it forms the largest fiber. It occupies the entire posterior of the ganglion's cell mass. Nucleus 2—Several injections. This mass is somewhat smaller, since its fiber is smaller. It lies immediately anterior to 1, but occupies only the ventral portion of the total mass. Nucleus 3—Three injections. A smaller elongated group of cells lying dorsal to 2. Nucleus 4—One injection. A small cell group at the antero-dorsal tip of the extraganglionic cell mass. Nucleus 5—No satisfactory injection. One attempt. Nucleus 6—One injection. About the same size as 4 and lying just medial to it. Nucleus 7—One injection. A small group just anterior to 2 on ventral side of the whole cell mass. Nucleus 8—No satisfactory injection. Three attempts. Nucleus 9—One injection. A very small elongated group of cells lying ventral to 6.

* This work was supported by a grant from the National Foundation for Infantile Paralysis.

was pushed at 30-40° angle directly through the mantle muscle to penetrate the giant fiber at some point in its course. Dye was injected by slight pressure through a closed water column attached to a 5 cc syringe. Injection was continued until the dye reached the nuclear mass in the stellate ganglion or, in the case of the second order fiber into the brain, until approximately 10 mm³(2) of dye was injected. Injected specimens were fixed in 15% formalin, dehydrated in alcohol, cleared in xylene, and imbedded in paraffin to be sectioned later.

Results. 1) By injecting each of the giant fibers of the mantle, it was possible to outline the cells of origin of each in the stellate ganglion. The diagrams show a ventral view and side view of the squid's right-side stellate ganglion, and map out each of the cellular masses except the fifth and eighth, for which no satisfactory results were obtained. When the same fiber was injected in different specimens the nuclear mass was always constant in location.

2) The nature of the giant synapse in the stellate ganglion, although already known, is easily demonstrated by the intracellular injection method. By injecting one of the mantle axons with 0.5% Trypan Blue to its nuclear origin and then exerting slightly more pressure, the interaxonal plasma membranes can be ruptured and all the giant axons filled with bright blue dye. Then 1% Trypan Red is injected into the second order fiber, whose end feet form the giant synapse. When viewed

through the low power dissecting microscope, the giant synapse is shown in brilliant red and blue. There is no admixture of dye, showing that the axon end feet are separated from the giant fiber axons by some form of membrane.

3) The cells of origin of the second order fiber in the brain, previously unknown, were easily located by gross inspection; however, finer localization must await microscopic sections. The second order fiber leaves the stellate ganglion and penetrates the muscular body wall, traveling in a nerve bundle along the dorsal side of the body cavity to pierce the cartilaginous brain case almost at the midline, just dorsal to the esophagus. Within the brain the fiber bends sharply laterally and ventrally and ends after completing a hemi-circle in a small ventrolateral lobe behind the large optic lobe.

Summary. 1. A new intracellular staining method is proposed as a way of obtaining definitive knowledge as to the location of single fiber nerve tracts, their connections, branches and cells or origin. 2. The method is applied to the giant fibers of the squid with definitive results.

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Development of a Strain of Spontaneously Hypertensive Rabbits.* (21255)

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Essential hypertension develops in man without demonstrable renal, neural, or vascular involvement and becomes more severe in

older age groups. Hamilton, *et al.* believe that essential hypertension is not a disease entity but merely a condition seen in that section of the population presenting arterial pressures higher than an arbitrarily selected value(1). Without a definite disease to which they can attribute the elevated pressure, in-

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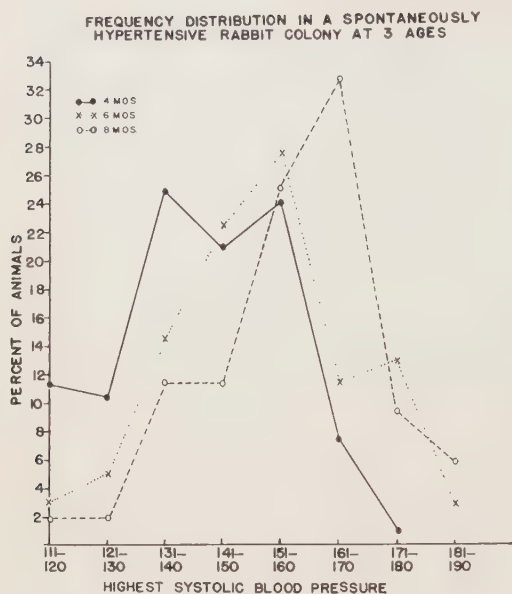


FIG. 1.

investigators have looked to emotional factors and environmental stresses as possible causes for hypertension.

An hereditary or familial trait has long been considered a predisposing factor in the development of essential hypertension(2,3). Knowledge about this particular component of essential hypertension has come mostly from clinical data based on family histories. The possibility of a controlled study of the inheritance of hypertension was presented to us when we noted that occasional stock rabbits had elevated pressures, *i.e.*, spontaneous hypertension. This paper deals with the results obtained from breeding such animals.

Method. New Zealand White, Dutch, and Californian rabbits were used. The incidence of spontaneous hypertension in these rabbits is low. This necessitated going directly to rabbitries in the Southern California area where large populations of rabbits could be screened. Out of 553 rabbits which were screened, 2.7% had systolic pressures of 160 mm Hg or more on 2 separate occasions and were selected as breeding stock. Their offspring cared for in a special rabbitry constructed for the purpose so that the environmental conditions were kept standard. We started with 8 separate matings, then interbred within the same family,

and also introduced new spontaneously hypertensive animals into the original groups. Blood pressure from the abdominal aorta was determined with a sphygmomanometer cuff. This method has been described in detail elsewhere (4). The average of 4 or 5 systolic and diastolic determinations was recorded. We have obtained where possible the blood pressures on each rabbit that was born at our rabbitry for a period of at least 8 months. Readings can be obtained at 3 to 4 months of age (4 to 6 lb weight) and were taken as often as once or twice a month thereafter. For comparison with the cuff values, intra-arterial readings have been made on some of these animals. Two per cent procaine was infused locally and blood pressure obtained from the femoral artery with a No. 24 needle attached to a Satham strain gauge(5).

Results. Fig. 1 and Table I show the frequency distribution and mean values of the highest systolic pressure obtained from each animal bred from spontaneously hypertensive parents within the indicated age groups. The mean systolic pressures at 5 to 6 and 7 to 8 months of 155 and 160 mm Hg, respectively, are each statistically significant when compared to the mean of 145 mm Hg at 3 to 4 months. (The reason less animals were available for the 6 and 8 month groups is that they died from intercurrent diseases after 4 months. One family of 6 rabbits was eliminated after 6 months because of very low pressures; at the time, we did not wish to keep them. Otherwise, no selection was made for compilation of the statistical data.)

Data on normal rabbits have been reported by others. McGregor found the average abdominal systolic pressure to be 125 Hg with a range of 90 to 160 mm Hg in 1120 readings on 84 normal rabbits(4). These animals were probably 3 to 4 months old since that is the usual age of the ordinary laboratory rabbit. In our own laboratory, 4-month-old animals average 135 mm Hg with a range of 120 to 150 after one year. These animals show an average increase of about 10 mm Hg in systolic pressure(6).

Fig. 2 shows the relationship in 30 rabbits between systolic pressure obtained with the abdominal cuff and by direct femoral artery

TABLE I. Distribution of Highest Abdominal Systolic Blood Pressure for Entire Spontaneously Hypertensive Colony of Rabbits. (Some rabbits are represented in more than one of the age groups.)

Age (mo)	3-4		5-6		7-8	
No. of rabbits	96		62		52	
Systolic pressure, mm Hg	No. of rabbits	% of total No.	No. of rabbits	% of total No.	No. of rabbits	% of total No.
121-150	65	68	28	45	14	27
151-160	23	24	17	27	13	25
161-185	8	8	17	27	25	48
\bar{X} *	145		155		160	
σ †	± 15		± 16		± 15	

* \bar{X} —Arithmetic mean of systolic blood pressures obtained from frequency distribution, $\bar{X} = \frac{\sum fX}{N}$.

† σ —Stand. dev. (mm Hg) = $\sqrt{\frac{f(X^2)}{N}}$.

puncture. In each case, the cuff pressure was taken just preceding the dissection and puncture for the direct determination. The cuff pressures average 13 mm Hg above the direct pressure with a range from 0 mm Hg to 30 mm Hg. Only in 3 cases was the direct reading higher than the cuff value. This relationship exists in normal rabbits also(6).

The spontaneously hypertensive rabbits do not maintain a high steady pressure. An example of fluctuation in pressure is seen in

Table II. This is the protocol of a typical offspring of spontaneously hypertensive parents. Normal rabbits also show variations in abdominal systolic pressures(4).

A frequency distribution of abdominal systolic pressures was made for the 2 sexes. The mean values were not significantly different between the two sexes at any of the 3 age groups. However, the mean systolic pressures suggest that females may have a greater change of pressure than males over an 8 month period (Table III).

There was no correlation between body weight and systolic pressure in any single age group. Naturally, as the animals reached 8 months, the average body weights were higher than at 4 months of age. However, certain rabbits did not gain weight as rapidly as others, but still had high blood pressure at 8 months of age.

Discussion. Our results have shown that offspring of spontaneously hypertensive rabbits have a high incidence of elevated systolic pressures, compared to the normal rabbit population. Three characteristics of these spontaneously hypertensive rabbits are worthy of note. First of all, the mean systolic pressure values for each of the 3 age groups mentioned are not extremely high. When compared to animals made hypertensive by various experimental methods, our colony has only moderately elevated pressures(7). Secondly,

COMPARISON OF ABDOMINAL CUFF PRESSURE AND FEMORAL ARTERY PRESSURE IN 30 ANIMALS FROM SPONTANEOUSLY HYPERTENSIVE RABBIT COLONY

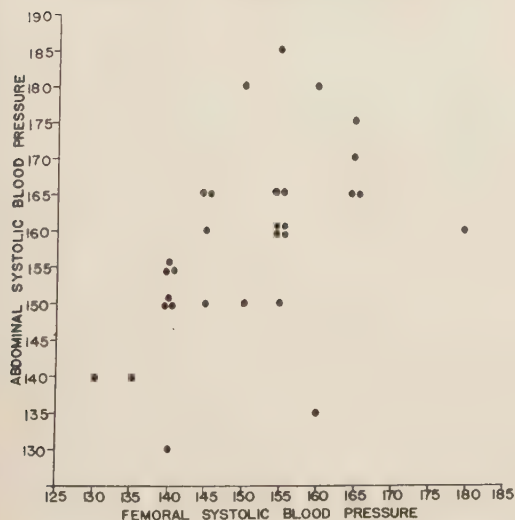


FIG. 2.

the pressures tend to fluctuate in spontaneously hypertensive rabbits. This is also true of normal rabbits, whereas rabbits with experimentally induced hypertension have more stable pressures. Lastly, the mean systolic pressure for our colony becomes significantly higher at 6 or 8 months compared to the mean value at 4 months. Normal rabbits also show a tendency to have higher pressures as they get older. The ordinary New Zealand White laboratory rabbit is considered adult at 4 months at which time it can be reproductive. It continues to grow and gain weight for the first 6 months to a year of its life. However, it can be stated that some animals in our colony which did not continue to gain weight as rapidly as others, tending instead to level off at 4 or 6 months, nevertheless had increasing blood pressure with age.

Summary. We have bred and raised a colony of spontaneously hypertensive rabbits.

TABLE II. Typical Protocol of Spontaneously Hypertensive Rabbit.

Pd-3 (male) Born: 7/11/53			
Date	Wt (lb)	1st reading	2nd reading (5 min. later)
10/15/53	5½	150/105	150/100
11/ 7	6¼	170/130	165/120
11/30	6½	170/135	160/125
1/13/54	6½	135/ 90	140/100
1/28		Cuff pressure:	160/110
		Femoral puncture:	150/115

TABLE III. Mean Systolic Pressures of Female and Male Rabbits in the Spontaneously Hypertensive Colony.

Age (mo)	3-4		5-6		7-8	
	♀	♂	♀	♂	♀	♂
No. of animals	43	53	29	33	23	29
Mean systolic pressure, mm Hg	145	150	155	160	165	160

It is concluded that there is a hereditary factor in hypertension and that the characteristic changes of blood pressure in spontaneously hypertensive animals are similar to the normal rabbit population. These findings would indicate that spontaneously hypertensive rabbits have characteristics in common with man in his development of essential hypertension.

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Effect of Dietary Aminopterin and Sulfasuxidine on Biosynthesis of Ascorbic Acid in the Rat.* (21256)

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Williams(1) reported that dietary aminopterin markedly reduced liver ascorbic acid concentrations in the rat. Subsequently, Schwartz and Williams(2) observed that the

depression in liver ascorbic acid was not the result of an increased rate of urinary excretion of this vitamin. It has been further reported (3) that succinylsulfathiazole (sulfasuxidine) when fed at a 2% level in a purified diet also has a depressant action on rat liver ascorbic acid.

In the present work we have investigated various possible methods by which amino-

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TABLE I. Ascorbic Acid Catabolism in Liver Homogenates* from Normal and Aminopterin-Fed Rats. (2-hr incubation at 37°C.)

Ration of animals	No. of exps.	Ascorbic acid in $\mu\text{g}/\text{flask}$			% destruction of ascorbic acid (b/a \times 100)
		(a) Avg initial conc.	(b) Avg final conc.	Avg destruc- tion of as- corbic acid	
Basal†	7	1033	757	276	26.7 (14.2-36.5)‡
Basal + aminopterin	6	1004	807	197	19.4 (11.3-32.0)

* Detailed flask contents are given in text.

† Avg destruction of ascorbic acid in Krebs-Ringer phosphate buffer alone was 3.6% with a range of 0-9.1%.

‡ Range of values.

pterin and sulfasuxidine may depress liver ascorbic acid. Four major possibilities exist: 1) Aminopterin and sulfasuxidine may inhibit the biosynthesis of ascorbic acid in the rat; 2) they may deplete the liver of ascorbic acid by inducing an abnormally high excretion of the vitamin; 3) they may increase the rate of ascorbic acid catabolism in the liver; or 4) they may cause a translocation of ascorbic acid so that, while the liver is depleted of the vitamin, other organs are enriched with it. The direct verification of the first hypothesis is difficult because as yet no adequate *in vitro* system for demonstrating ascorbic acid synthesis in animal tissues has been reported. Previous work(2) has shown that the second hypothesis is not valid since there is no abnormally high urinary excretion of ascorbic acid in aminopterin-fed rats. If the 2 remaining hypotheses could be ruled out, the effect of aminopterin and sulfasuxidine on rat liver ascorbic acid could probably be explained by the first hypothesis, *i.e.*, that these substances exert their effects on the biosynthesis of ascorbic acid. In the present paper, results obtained in studying these possible mechanisms by which aminopterin and sulfasuxidine decrease liver ascorbic acid in the rat are reported.

Experimental and results. Effect of dietary aminopterin on ascorbic acid catabolism in rat liver homogenates. Adult male rats of the Sprague-Dawley strain were used as experimental animals. One group of rats was fed a basal ration consisting of 18% casein, 4% salts IV(4), 5% corn oil, 2% vitamin mixture (5) which was supplemented with one μg of vit. B₁₂ per g of mix, and 71% sucrose. An-

other group was fed the basal ration to which aminopterin was added at a level of 4 mg/kg of ration. After about 2 weeks the group receiving aminopterin exhibited the typical aminopterin toxicity syndrome: porphyrin secretion around the eyes and nose, diarrhea, and general inactivity leading to death. When these symptoms appeared, the rats were sacrificed and 1:4 liver homogenates in Krebs-Ringer phosphate buffer, pH 7.4, were prepared. Twenty-five ml Erlenmeyer flasks containing one ml of liver homogenate from control or aminopterin-fed rats, one ml of Krebs-Ringer phosphate buffer, 0.5 ml of freshly neutralized ascorbic acid solution (2 mg/ml), and 0.5 ml of distilled water were incubated at 37°C. One ml aliquots of the reaction mixture were taken at the beginning of the incubation and again after 2 hours. After deproteinizing with 5% metaphosphoric acid in 10% acetic acid, the aliquots were analyzed for ascorbic acid by the method of Roe and Keuther(6) as modified by Bolin and Book(7). A further modification by us included the substitution of a mixture of 3 parts concentrated HCl and 2 parts of phosphoric acid (85% by volume) for the 85% sulfuric acid of the original method. The results of this experiment are presented in Table I. It should be noted that the osazone method of ascorbic acid analysis, which was used here, detects ascorbic acid, dehydroascorbic acid, and diketogulonic acid. This means that the disappearance of ascorbic acid registered by this method indicates the amount of ascorbic acid which has been degraded beyond diketogulonic acid, presumably to compounds arising from a rupture of the carbon chain. Since the

TABLE II. Effect of Dietary Aminopterin on Liver, Adrenal, Spleen, and Kidney Ascorbic Acid.

Organ	Basal group		Aminopterin-fed group	
	Asc. acid conc. in $\mu\text{g/g}$ organ	Total organ asc. acid in μg	Asc. acid conc. in $\mu\text{g/g}$ organ	Total organ asc. acid in μg
Liver	359* (318-426)†	Not d‡	173 (132-252)	Not d‡
Adrenal	4431 (3647-5979)	67 (40-83)	3241 (1298-4411)	68 (40-110)
Spleen	579 (421-657)	446 (349-582)	601 (420-772)	290 (176-333)
Kidney	183 (164-218)	192 (161-234)	120 (90-160)	109 (76-168)

* Each figure represents avg value obtained from analysis of 5-7 organs.

† Range of values.

‡ Not determined. Other studies showed that livers of aminopterin-fed rats are somewhat smaller than those of rats fed a basal ration. Therefore, the size of the livers could not compensate for the decreased ascorbic acid concentrations.

data of Table I indicate that ascorbic acid in Krebs-Ringer phosphate buffer is stable at 37°C for 2 hours, the destruction observed upon incubation in the presence of liver homogenates must be effected by the homogenates. The table indicates that there is slightly less destruction induced by liver homogenates from aminopterin-fed rats than that induced by control rat liver homogenates. Therefore, it is clear from the data that dietary aminopterin does not cause an abnormally high metabolic breakdown of ascorbic acid.

Effect of aminopterin on the translocation of ascorbic acid within the rat. The same rats used in the work described above were also utilized for this study. The ascorbic acid content of the liver, spleen, kidney, and adrenal gland of both groups of rats was determined. The spleen, kidney, and adrenal weights were recorded and these organs were homogenized in 5% metaphosphoric acid-10% acetic acid mixture. The deproteinized extracts were analyzed for ascorbic acid by the modified method of Roe and Keuther noted above.

In Table II are presented both ascorbic acid concentrations and values for the total organ ascorbic acid content. Previous results indicating decreased liver ascorbic acid in aminopterin-fed rats(1,2) have been confirmed by these data. From the table it can also be seen that aminopterin depresses both adrenal and kidney ascorbic acid concentrations but has no significant effect on spleen ascorbic acid concentrations. However, the total spleen as-

corbic acid content of the aminopterin-fed group was considerably lower than that of the control group. This was due to the large difference in the weight of the spleens of the 2 groups of rats. The difference in average adrenal weights also explains why the total adrenal ascorbic acid in both groups is approximately equal. Since there is no significant increase in spleen, kidney, or adrenal ascorbic acid, it can be concluded that the decrease in liver ascorbic acid is not associated with a translocation of this vitamin from the liver to other organs in the rat. Since the possibility of increased urinary excretion of ascorbic acid in the aminopterin-fed rat has previously been ruled out(2), and the present experiments invalidate the increased catabolism and translocation possibilities, the first hypothesis, namely, that aminopterin inhibits the biosynthesis of ascorbic acid, is considerably strengthened.

Guinea pig experiments. If aminopterin and sulfasuxidine inhibit the biosynthesis of ascorbic acid, then upon feeding these compounds to guinea pigs which receive an adequate dietary supply of ascorbic acid, no depression in the liver levels of ascorbic acid should occur. Therefore, the effect of these 2 substances on liver ascorbic acid levels in young, male guinea pigs was studied. In the first experiment, one group of guinea pigs was fed the purified ration of Roine, Booth, Elvehjem, and Hart(8), while a second group received the same ration plus an oral supple-

TABLE III. Influence of Dietary Aminopterin and Sulfasuxidine on Liver Ascorbic Acid Levels in Guinea Pigs Receiving Adequate Dietary Ascorbic Acid.

Diet fed	Exp.	Feeding period	No. of animals	Liver ascorbic acid, $\mu\text{g/g}$
Basal	I	10-12 days	6	51 (27- 79) *
" + aminopterin		10-12 "	5	78 (51- 98)
Basal	II	10 "	7	39 (21- 51)
" + aminopterin		10 "	5	37 (15- 92)
Basal	III	6 wk	7	73 (44-104)
" + sulfasuxidine		6 "	8	77 (42-108)

* Range of values.

ment (by pipette) of 3 mg of aminopterin per guinea pig per day (9). To insure an adequate dietary intake of ascorbic acid, this vitamin was administered to both groups at a level of 10 mg of ascorbic acid/guinea pig/day. After about 2 weeks the aminopterin-fed guinea pigs exhibited a marked decline in weight, which within a few days terminated in death. This weight loss was also associated with leukopenia as demonstrated by leukocyte counts performed on blood removed from the ears of the animals. During this period of rapid weight loss, the guinea pigs were killed and the livers removed for ascorbic acid analysis.

The results of the first experiment, Exp. I, are presented in Table III. It can be observed that there was no depression of liver ascorbic acid induced by orally administered aminopterin. A repetition of this experiment (Exp. II) gave essentially similar results although the absolute values obtained in this second experiment were lower than those previously obtained.

In the third experiment, the guinea pigs were fed sulfasuxidine at a level of 2% in the purified ration of Roine *et al.* From Table III it can be seen that sulfasuxidine, after a 6-week feeding period, also had no effect on liver ascorbic acid in the guinea pig.

The above findings which show that there is no depression in guinea pig liver ascorbic acid induced by dietary aminopterin or sulfasuxidine is considered as added evidence that the decrease in liver ascorbic acid observed in rats fed either of these 2 compounds is due to an inhibition of the biosynthesis of ascorbic acid in the rat.

Discussion. Recently, Sauberlich(19) has

confirmed our earlier findings with respect to the aminopterin-induced depression in liver ascorbic acid in the rat. Sauberlich has further reported that the *Leuconostoc citrovorum* factor overcomes this depressant effect of aminopterin on liver ascorbic acid. Therefore, aminopterin, in lowering liver ascorbic acid, apparently acts in its well-known role as a folic acid antagonist. The action of sulfasuxidine is more obscure. We have found that sulfasuxidine exerts its depressant effect even in the presence of folic acid(3).

Our results indicate that both sulfasuxidine and aminopterin inhibit the biosynthesis of ascorbic acid, although the major portion of the present studies were concerned with aminopterin. The effect of sulfasuxidine has been dealt with extensively in a previous communication(3). If sulfasuxidine acted to depress liver ascorbic acid in the rat in any manner other than inhibition of biosynthesis, a depression in guinea pig liver ascorbic acid would be expected. As reported above, this depression was not found.

Sulfasuxidine is very poorly absorbed through the intestinal wall(10). This means that the inhibition of ascorbic acid biosynthesis in the rat by sulfasuxidine, and perhaps by aminopterin also, probably occurs in the intestinal tract. Since aminopterin and sulfasuxidine are bacterial inhibitors(11-15), they may exert their depressant action on liver ascorbic acid by inhibiting the growth of certain intestinal micro-organisms which produce some necessary component of the ascorbic acid synthesis system. With respect to this possibility, it is interesting to note that Reyniers(16) has reported that germ free suckling rats require a dietary source of ascorbic acid.

Aminopterin and sulfasuxidine have also been reported to cause morphological changes in the intestinal wall(17,18); and they may act, then, by interfering with some reaction taking place there, a reaction which is vital in the chain of reactions ultimately producing ascorbic acid.

Summary. Possible mechanisms by which dietary aminopterin and sulfasuxidine decrease levels of liver ascorbic acid in the rat have been investigated. In previous studies an increased rate of ascorbic acid excretion after feeding aminopterin had been found not to occur. In the present studies, dietary aminopterin has been found not to induce an increased destruction of ascorbic acid by liver homogenate or a translocation of liver ascorbic acid to other organs. Also, aminopterin and sulfasuxidine do not depress liver ascorbic acid in guinea pigs receiving an adequate daily supply of ascorbic acid. Therefore, all of these findings are considered as evidence that aminopterin and sulfasuxidine decrease liver ascorbic acid of the rat by inhibiting the biosynthesis of the vitamin. Possible mechanisms of this inhibition are discussed.

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Influence of Photodynamic Effects on Diffusion in Rabbit Dermis.* (21257)

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Castellani(1) stressed the importance of an unknown photodynamic effect on mucopolysaccharides of connective tissues, namely hyaluronic acid. *In vitro* he observed a steady reduction of viscosity of hyaluronic acid mixed with hematoporphyrin and exposed

to visible light, as compared with hyaluronic acid mixed with hematoporphyrin and not exposed to light. According to the author, such reduction of viscosity is due to a depolymerization of this mucopolysaccharide.

In view of the importance which this phenomenon might have for interpretation of the pathogenesis of cutaneous diseases caused by

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TABLE I. Diffusion Areas after Intradermal Injection of 0.1 ml of a Mixture of Equal Amounts of Haematoporphyrin and India Ink. Six animals.

Diffusion surfaces in mm ²		
Exposed to light	Unexposed	Δ
209	45	164
118	52	66
388	71	317
220	108	112
98	26	72
77	25	52
Mean 185	54	131

Δ Difference between surfaces in mm².

photodynamic effects, it seemed useful to investigate it *in vivo*. The following materials have been used: A) hematoporphyrin (prepared by the Nordmarck Werke, Hamburg) at 5% dilution in distilled water. B) India ink (Pelikan trade-mark of the Günther Wagner firm). C) diphtheria toxin (prepared by the Istituto Sieroterapico e Vaccinogeno Sclavo of Siena).

Methods. Experiments were carried out on albino rabbits of the same breed, weighing approximately 2 kg each. The above mentioned substances were intradermally injected in the rabbits' back after careful shaving. The animals were inoculated in the left side and subsequently exposed to light; control injections were symmetrically given on the right side. Six rabbits received, both in the right and left sides, two 0.1 ml injections (one side each) of a suspension containing equal parts of A (hematoporphyrin) and B (India ink). The right side was covered with a wet cloth, and a black sheet of paper was fixed along the spine. The left side was exposed to

the light of 3 electric bulbs for a total of 1600 W at 15 cm distance. The exposed area was then cooled by means of ventilator draught and moistened every 3 minutes so that the cutaneous temperature should never exceed 28-30'. The exposure to light went on for 2 hours, then the areas where the inoculated material had diffused were outlined in ink and traced on cellophane. Measurements were recorded on coordinate paper in order to have the surface value in mm². Data of this series are shown in Table I. Two rabbits were given 2 injections a side of 0.2 ml of a solution made with equal amounts of hematoporphyrin and diphtheria toxin: the dry toxin contained in an ampoule was dissolved in 0.5 ml of saline, which yielded a concentration 6 times higher than is normally used for the Schick reaction. The left side was exposed to light as the other series, the right side being protected against light. After 2 hours' exposure to light, the diffusion area (which was clearly visible) of hematoporphyrin was measured. After 24 hours the area of initial central necrosis and surrounding inflammatory edema was recorded. After 48 hours the area of both the edema and the necrotic hemorrhagic lesion was again recorded. Figures for this series of experiments are shown in Table II. To check whether a variation of diffusion might be due to any cause other than exposure to light, 2 rabbits were inoculated in both flanks with 0.2 ml of India ink, the left side only being exposed to light, protection against light being equally provided for the right side. To the same purpose another rabbit was inoculated in both sides with 0.2 ml of diphtheria toxin, its concentration being half that

TABLE II. Inflammation Areas after Intradermal Injection of 0.2 ml of a Mixture of Equal Amounts of Haematoporphyrin and Diphtheria Toxin.

Animal No.	2 hr*			24 hr*			48 hr*		
	A	B	Δ	A	B	Δ	A	B	Δ
1	130	28	102	655	128	527	1280	433	847
	100	25	75	550	64	486	570	350	220
2	99	40	59	1492	133	1359	942	263	679
	96	21	75	1370	162	1208	1283	360	923
Mean	106	28	78	1016	121	895	1018	351	667

* Time elapsed between inoculation and measurement of inflammation area.

A—Inflammation area in skin exposed to light.

B— " " " normal skin.

Δ —Difference between surfaces in mm².

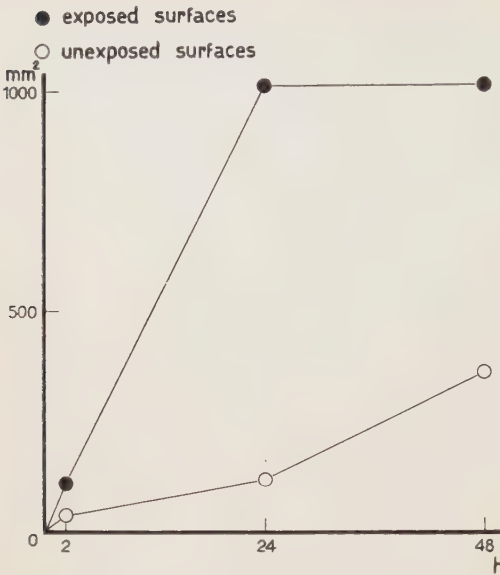


FIG. 1. Mean surface value of inflamed areas inoculated with haematoporphyrin and diphtheria toxin.

used for the previous group treated with hematoporphyrin and toxin: the final toxin concentration being therefore the same. The left side was exposed to light while the right side was protected.

Results. Besides the data shown in the tables, it should be pointed out that in the group treated with India ink and hematoporphyrin the diffusion areas of unexposed skin showed clear-cut margins, whereas the exposed skin areas were more irregular and less sharply defined. On the controls of the group treated with diphtheria toxin and hematoporphyrin the typical necrotic lesion surrounded by slight edema was clearly seen within 24-48 hours. On the contrary, the exposed skin lesions showed less necrosis, whereas the surrounding edematous area was wider and highly hyperemic. At 72 hours the necrotic lesion of the unexposed side was quite evident and remarkably large whereas that of the exposed side was much smaller with more irregular edges; the edematous zone had practically disappeared. The 2 control rabbits inoculated with India ink only, showed no appreciable difference between the skin spots that had been exposed to light and those that had been protected. No difference was observed between

the exposed and the unexposed skin spots of the control rabbit inoculated only with diphtheria toxin.

For the interpretation of the phenomena observed, some reference seems indicated to the many recent observations which emphasize the relationship between the spreading power of some substances (bacteria, viruses, dyes, toxins, etc.) inoculated into the skin, and the state of the connective tissue ground substance. The spreading effect after destruction of the ground substance by either hyaluronidase or the action of various chemical substances (ascorbic acid, azoproteins) is well known. It has also been demonstrated that the degree of polymerization of the ground substance is in relation to the effect of various hormones (Opsahl, White, and Duran-Reynals(2); Lurie and Zappasodi(3); Catchpole, Gersh, and Pan(4) which thus gain more importance also for what concerns the diffusion phenomena on the ground substance. On the other hand, the above mentioned possibility of hyaluronic acid being depolymerized *in vitro* by a photodynamic action has been recently demonstrated. Since it is well known that luminous radiations easily find their way through the epidermis, it seems reasonable that the increased spreading power observed in the experiments here reported is the result



FIG. 2. Mean surface value of inflamed areas inoculated with haematoporphyrin and India ink.

of an *in vivo* photodynamic effect on the ground substance. The phenomena of increased diffusion here described should be therefore considered as being of the same type as those observed whenever the degree of polymerization of the ground substance components, particularly hyaluronic acid, had been altered by either chemical or enzymic agents.

Summary. As a result of a photodynamic effect, a constant notable increase has been observed in the diffusion of India ink and diphtheria toxin inoculated in the dermis. Since the spreading test may be assumed, to

a certain extent, an indication of the conditions of the ground substance of dermal connective tissue, these results seem to demonstrate that after a photodynamic effect this substance is modified.

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Tests for Isoantibodies Active in Conjunction with Guinea Pig Serum Against 6C3HED Lymphoma Cells.* (21258)

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In previously reported experiments, normal guinea pig serum brought about the death of transplanted 6C3HED lymphoma cells when injected intraperitoneally into mice carrying them but had no effect on the lymphoma cells during many hours' contact at 37°C *in vitro*, the findings showing plainly that the effect *in vivo* was the result of some reaction in which the guinea pig serum and the animal host both participated(1). Since the C3H mice used as host animals occasionally manifested some resistance against the transplanted 6C3HED lymphoma cells—this being engendered presumably by antigens present in the long-transferred lymphoma cells but absent from the tissues of the current hosts, and related perhaps to the presence of isoantibodies in the blood of the resistant animals (2,3)—the possibility was considered that the resistant animals might provide isoantibodies which, acting in concert with the guinea pig serum, killed the transplanted 6C3HED lymphoma cells *in vivo*(1). The fact that mouse serum is normally devoid of one or more of

the constituents of hemolytic complement(4) has interest in relation to this possibility, and a recent observation seemed to lend additional weight to it. For guinea pig serum, while inhibiting to some extent the cells of an AKR lymphoma that had been transplanted for some time in AKR mice of the line in which it originated, with transfer later to AKR mice of another (presumably to some extent resistant) line, had little or no effect *in vivo* on the cells of several AKR lymphomas that had been transferred only a few times in mice of the inbred line in which they had arisen (and which were presumably not resistant to the cells of the respective lymphomas)(5).

If isoantibodies were the host factors that participated with the injected guinea pig serum in bringing about the *in vivo* effects referred to above, their presence should be disclosed directly by experiments in which mouse serum containing the isoantibodies is mixed with active guinea pig serum, the mixture then being held in contact with the lymphoma cells during suitable periods of time *in vitro*, with tests made later for viability of the exposed cells. Such experiments are feasible and a number of them have been done, as will now be described.

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Experimental. The hypothetical isoantibodies might conceivably be found in the serum of normal C3H mice of the inbred line used, though previous work done by others does not make this seem likely(2,3). If such antibodies are actually the responsible host factors, they would have to be present in the serum of all the C3H mice carrying 6C3HED lymphomas or implanted with the lymphoma cells—or else be very rapidly engendered in such animals following injection of the normal guinea pig serum; for much experience has shown that the 6C3HED lymphoma cells regularly begin to die within a few hours after normal guinea pig serum has been injected into animals carrying them, and this proves true even in animals in which a single injection of guinea pig serum is given one hour after implantation with the cells(1). Furthermore, the hypothetical isoantibodies might be expected to be present in high titer in the serum of mice that had recently overcome the growths, especially if these resistant hosts were of an alien breed(2). Hence in the present work tests were made with 1 or more specimens of pooled serum procured from mice in each of the following categories: normal C3H mice (bred in this laboratory), C3H mice with enlarging 6C3HED lymphomas, C3H mice from which 6C3HED lymphomas had regressed and which had recently proved negative to challenge reimplantations with large numbers of the lymphoma cells, normal strain A mice (regularly resistant to the 6C3HED lymphoma cells), and strain A mice in which 6C3HED lymphomas had regressed and which had recently proved negative upon challenge re-implantation.

In each experiment, mixtures were set up containing three ingredients, as follows: A) 6C3HED cells, freshly suspended as individuals in buffered-glucose-Ringer's solution, as described in detail elsewhere(1), in a final concentration of 4,000 cells/mm³. B) Specimen of pooled mouse serum to be tested for isoantibodies. This was regularly procured from 9 or more mice; usually the specimen was tested the day of bleeding, though occasionally it had been kept frozen for a few days at -22°C. The specimen was used in a final dilution of 1:4. C) Normal guinea pig

serum (pooled specimens from at least 12 animals; kept frozen at -22°C for periods up to 4 weeks; known from subsidiary tests to be active against 6C3HED lymphoma cells *in vivo*). This was likewise regularly used in a final dilution of 1:4; alone it did not harm the cells in any of the present experiments, as will be mentioned again further on, while in the previously reported experiments whole guinea pig serum did not harm 6C3HED lymphoma cells when held in contact with them for 6 hours at 37°C(1). Control mixtures were included to test the viability of the cells kept under the actual conditions of each experiment, and for any effects the guinea pig serum itself might have on the lymphoma cells.

The mixtures were incubated 4 hours at 37°C, then gently centrifuged with removal of virtually all the supernatant liquid and resuspension of the lymphoma cells in the Ringer's solution buffered at pH 7.4 to which glucose 2 mg per cc had been added. Approximately 2 million of the resuspended lymphoma cells were then implanted into each flank of 3 susceptible C3H mice, with careful observation of the test animals afterwards. To insure that the *in vitro* conditions were such that any antibodies present in the test serums would have a favorable chance to act in conjunction with the guinea pig serum, 2 additional control mixtures were included in 4 experiments. These mixtures both contained, in addition to the known number of 6C3HED lymphoma cells, an antilymphoma immune serum made by injecting the 6C3HED lymphoma cells together with Freund's adjuvants into 6 rabbits and pooling the serum, which was harvested 3-6 weeks after the immunizing injections. In one of these mixtures, the immune serum was used alone, in a final dilution of 1:64; in this concentration, the immune serum was regularly devoid of effect on the lymphoma cells. In the other mixture the immune serum was used in a final dilution of 1:64, together with guinea pig serum in a final dilution of 1:4; this mixture regularly killed all the lymphoma cells with which it was held in contact in the 4 experiments.

Findings. Nine experiments were done as just described. In these, tests were made with

7 specimens of pooled serum from normal C3H mice, with 5 specimens of pooled serum from C3H mice with enlarging 6C3HED lymphomas (resulting from implantations made 5 to 14 days before bleeding), with one specimen from 10 C3H mice that had once overcome 6C3HED lymphomas and resisted challenge re-implantations made 11 days before bleeding, with 5 specimens from normal A mice, and with 5 specimens from A mice that had overcome 6C3HED lymphomas and proved resistant to re-implantation with 6C3HED cells 8 days before bleeding. In each experiment the lymphoma cells that had first been incubated in mixture with the buffered-glucose-Ringer's solution, then centrifuged, resuspended, and implanted as described above, regularly formed palpable tumors within 7 to 10 days, and these grew progressively in the implanted test mice, usually bringing about death between the 20th and 30th days. The same was true of the cells that had been exposed to the effects of either guinea pig serum alone or immune rabbit serum alone. The lymphoma cells that had been exposed to the immune rabbit serum in mixture with guinea pig serum, however, regularly failed to grow, as has already been stated. The lymphoma cells that had been exposed to the effects of guinea pig serum in mixture with the specimens of pooled mouse serum mentioned above, all grew quite as readily as did the cells from the control mixtures, the findings providing strong evidence that none of the test specimens contained isoantibodies capable of killing the lymphoma cells *in vitro* in mixture with the guinea pig serum.

To pursue the matter further, an experiment was made to compare the effectiveness of guinea pig serum against the 6C3HED lymphoma cells *in vivo* in C3H mice of a "relatively susceptible" and a "resistant" strain, respectively. This was done to learn whether the guinea pig serum might have enhanced effectiveness in the resistant mice, on the assumption—in spite of the negative indication provided by the experiments already given—that the resistant animals might conceivably have isoantibodies, or be capable of developing these rapidly, in titer sufficient to prove effective against the 6C3HED lympho-

ma cells *in vivo*. C3H/JAX mice (procured from the Jackson Memorial Laboratory, Bar Harbor), were used as "resistant" mice. Recent experiments made in this laboratory have shown that these animals, when implanted with 2-4 million 6C3HED lymphoma cells in each groin, regularly develop palpable tumors within 7-10 days which enlarge notably during the ensuing week and are quite comparable in size and initial course to those developing in the "relatively susceptible" strain of C3H mice customarily used in this laboratory. These latter were originally procured from Dr. L. C. Strong and have been random-bred in this laboratory during the past several years; they are here referred to as C3H/CMC mice. More than 90% of the mice of this C3H/CMC strain that have been implanted with 2 million or more 6C3HED cells during the past year have developed progressively enlarging subcutaneous tumors and died with widespread lymphomatosis some 18 to 30 days following the implantations. In the C3H/JAX mice, however, the subcutaneous 6C3HED lymphomas have regularly regressed completely during the period 12 to 22 days following implantation, and the animals have remained healthy thereafter. In the experiment made to see whether guinea pig serum is more effective against the 6C3HED lymphoma cells in resistant mice, 4 groups of the C3H/JAX mice and 4 groups of the relatively susceptible C3H/CMC strain of mice (four animals in each group) were implanted with 2 million of the lymphoma cells in the subcutaneous tissue of each groin. One group of mice of each sort was then kept under observation without treatment as controls. One hour after implantation the mice of another group of each sort was given 2.0 cc of guinea pig serum intraperitoneally, and additional groups of each sort were given 1.0 and 0.5 cc, respectively. In the control mice of the relatively susceptible C3H/CMC strain, tumors almost 1.0 cm across were palpable on the 7th day following implantation; these growths enlarged progressively, killing 2 of the 4 mice on the 20th day and a third on the 22nd day, while the fourth animal was sacrificed when moribund on the 27th day. The growths of the control untreated mice of the

resistant C3H/JAX strain were quite comparable in size to those of the untreated C3H/CMC mice during the period 7 to 12 days following implantation. On the 11th day, for example, subcutaneous tumors measuring 13 to 21 mm across were present at each implantation site in the four hosts, and these growths were approximately the same size on the 12th day. On the 14th and 15th days, however, the growths were all much smaller, and by the 18th day they had disappeared entirely from all four mice. The guinea pig serum in general proved equally inhibitory in mice of the two sorts. Thus growths failed to appear in 3 of the 4 C3H/CMC mice given 2 cc of guinea pig serum, while small bilateral growths made a much delayed appearance in the fourth animal of this group; the same was true of the group of C3H/JAX mice given 2 cc of guinea pig serum, there being complete inhibition of the growths in 3 of the 4 animals, with delayed appearance of bilateral growths in the remaining one. 1 cc of guinea pig serum gave complete inhibition of the growths in 1 of the 4 C3H/CMC mice, with delayed appearance of growths in the other 3, while this amount of serum completely inhibited growth in two of the C3H/JAX mice and delayed the appearance of growths in the remaining two. The 0.5 cc of guinea pig serum likewise inhibited the growths markedly but incompletely in the relatively susceptible and resistant mice respectively, the results being quite comparable in the 2 groups. The findings as a whole made it plain that the inhibitory effects of the guinea pig serum was not notably enhanced in the resistant mice.

Summary. In the light of a previous observation—that normal guinea pig serum is highly effective against 6C3HED lymphoma cells *in vivo* but not *in vitro*, the host animal obviously participating in the reaction *in vivo*—a number of experiments were done to learn whether isoantibodies, capable of acting in conjunction with guinea pig serum against the lymphoma cells, might be present in the serum of normal mice of susceptible and resistant strains, in that of susceptible hosts carrying 6C3HED lymphomas, or in that of mice that had overcome implanted 6C3HED lymphoma cells and proved immune to re-implantation with them. The findings are given in some detail. They provide strong but not conclusive evidence that such isoantibodies are absent from the blood of mice in all the categories mentioned above. It follows that host factors or conditions of some other sort might profitably be sought to account for the participation by the host in the reaction whereby normal guinea pig serum, injected into animals carrying 6C3HED lymphomas, brings about death of the lymphoma cells.

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Relationship of Vitamin E to Embryonic Development of Avian Eye. (21259)

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The relationship of eye disorders to vit. E has been reported in a study of retrolental fibroplasia in human infants(1), and in an

eye disorder suggestive of retrolental fibroplasia in rats(2). Jensen(3) stressed the importance of vit. E in practical turkey rations in relation to embryonic mortality and hatchability. The effect of adding alpha-tocopheryl acetate to a practical type diet, with combina-

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tions of dried whey and fish solubles, on hatchability and embryonic mortality in Beltsville Small White turkeys will be reported herein.

Procedure. Seventy-two turkey hens were randomized into 8 groups of 9 hens each. These birds had been reared in electrically heated batteries with raised screen floors for 6 weeks, in standard growing batteries with raised wire floors from the 6th through the 12th week, and in pens with raised wire floors for the remainder of a 30-week period prior to the initiation of this experiment, as described in (4). The diet fed during the first 10 weeks consisted of 55% soybean oil meal, 20% ground yellow corn, 19% ground milo, 3% dicalcium phosphate, 2.5% ground oyster shell and .5% salt. This diet was supplemented with the following vitamins as indicated on a per lb basis: 2 mg riboflavin, 12.5 mg calcium pantothenate, 20 mg niacin, 400 mg choline chloride, 6 μ g vitamin B₁₂, 2 mg menadione, 160 mg manganese sulfate, 4500 I. U. vit. A and 1200 I. C. U. vit. D₃. From the 10th through the 30th week, soybean oil meal was decreased to 40% and ground yellow corn and ground milo were increased to 25 and 29% respectively. At the beginning of the present experiment soybean oil meal was decreased to 25% and ground yellow corn and ground milo increased to 35 and 34% respectively, and vit. A was increased to 9000 I. U./pound. The source of vit. A was a stabilized product which had been sealed in through the use of hydrogenated vegetable fat. Each group of 9 birds was placed in a 10' by 12' pen with a raised wire floor. Four nests were also placed in each pen. Feed and water were supplied *ad libitum*. Each pen of females was mated with a male of the same breed reared on the range of the Poultry Husbandry Department of the Texas Agricultural and Mechanical College System. Toms were rotated weekly to obtain maximum fertility. Groups 1, 3, 5 and 7 were fed the basal diet unsupplemented, supplemented with 5% fish solubles, with 3% dried whey and with 5% condensed fish solubles and 3% dried whey respectively. Groups 2, 4, 6 and 8 were fed the same diets except that the latter 4 mentioned groups also received a supplement of alpha-tocopheryl acetate at a level of

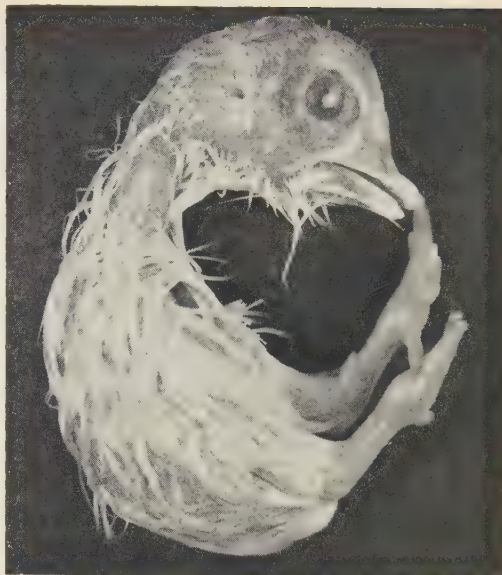


FIG. 1. Abnormal eye of a 21-day turkey embryo, from hen not supplied with supplementary alpha-tocopheryl acetate.

20 mg/lb. Eggs were collected at 4 stated intervals each day, were kept in a cooler at 55°F and were set once each week. Eggs were candled at 7, 14, 21 and 24 days. Infertile eggs and those containing dead embryos were broken to determine the approximate time of death. Eggs removed during incubation and those which failed to hatch on the 29th day of the incubation period were broken and a gross examination was made of the embryos. This report is concerned with the embryos that were at least 21 days of age or over. Live embryos were preserved in formalin or Zenker's fixative for later histopathological studies. Sections were stained with hematoxylin and eosin. Body measurements and skeletal measurements were also made of some of the embryos.

Results. Supplementation of the all-vegetable protein diet with fish solubles, whey, or a combination of these ingredients had no effect on the occurrence of the anomalies described herein.

A cloudiness in the central portion of the lens was found among the embryos from eggs laid by hens fed an all-vegetable protein diet without added vit. E. This condition was observed in both of the eyes in 13 of 30 live embryos examined on the 29th day. These

embryos pipped the shell but did not hatch. Seven of this number had hemorrhages in the vitreous humor. One or both of the eyes frequently had a bulging of the cornea, that gave the embryo a protruding eye appearance in extreme cases. The cornea was found to be slightly irregular in some embryos when examined with a binocular microscope. A yellowish-white spot of material was seen in the eyes between the lens and the cornea in a few specimens, as shown in Fig. 1, picturing a 21-day embryo that was alive when removed from the egg. This condition also occurred at later stages of embryonic development. An irregular-shaped iris was often associated with the bulging cornea, which made the pupil likewise irregular. Upon dissection of the lens, pressure applied firmly to the outside resulted in its rupture with a splitting out of a smaller miniature lens and the loss of a watery fluid. In some instances, a still smaller lens-like structure could further be forced out. The conjunctival blood vessels were dilated in many embryos and there were some with edema of the eyelids. The pecten was present but extensive observations on the pecten were not made. The eyes of the embryos obtained from dams supplemented with vit. E were apparently normal. The pupil was rounded, the iris evenly pigmented and the lens was clear. Dissection of the lens showed it to be a typical solid crystalline lens. The vitreous humor was clear.

The results of a preliminary histological examination of the lens from the 2 groups of embryos is shown by camera lucida drawings in Fig. 2. Fig. 2A is a section near the center of the lens of a 29-day embryo from a dam that was fed added vit. E. A portion of the iris and ciliary body are included. Fig. 2B is a similar section from near the center of the lens of an embryo of the same age where the dam did not receive supplemental vit. E. The lens is apparently the same diameter as 2A, but is more flattened and has a small lens body inside the outer lens layer. Degenerative changes have occurred throughout the lens, especially in the central and posterior portions. These observations are based on histological sections of both eyes from 5 of the 29-day embryos of the non-supplemented groups and the same number of embryos pro-

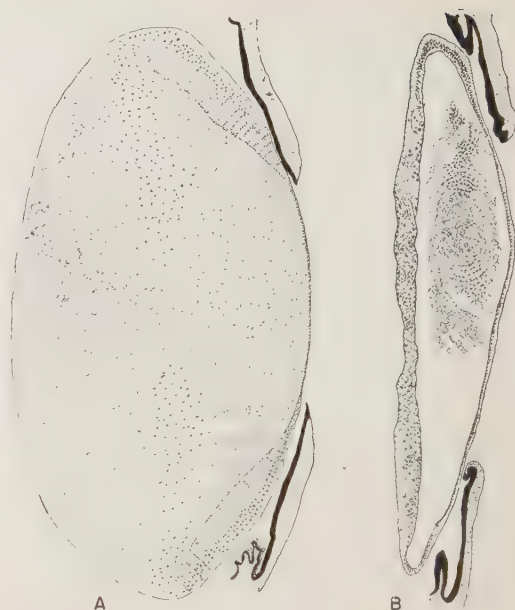


FIG. 2. Lens of turkey embryo (29 days); (A) from hen fed a supplementary source of alpha-tocopheryl acetate; (B) not supplemented.

vided supplemental alpha-tocopheryl acetate. Microscopic section of the eyes of a 24-day embryo from the unsupplemented group showed it to be as in Fig. 2B. Degenerative changes have also been observed in the retina. Further studies will be necessary to determine the extent of the degeneration and when such changes are discernible.

Measurements of the principal skeletal parts of live embryos from the supplemented and unsupplemented groups were made. These embryos were 29 days of age, and had pipped the shell but did not hatch. The legs, wings, and body length of the embryos from the groups not supplied additional alpha-tocopheryl acetate were less than the supplemented groups. The tarsometatarsus and toes of the unsupplemented groups were edematous, which made this part of the leg appear extremely shortened. Ten of the 30 live embryos examined from the unsupplemented groups on the 29th day of the incubation period also had an edematous area on the neck at the back of the head. This condition did not occur in the alpha-tocopheryl supplemented groups.

Caution should be observed in interpreting abnormalities in dead embryos. However, 71

dead embryos from dams fed the diet not supplemented with alpha-tocopheryl acetate were examined. The number of dead embryos for each day between the 21st and the 28th day were 1, 2, 1, 11, 6, 19, 15, and 16 respectively. Fifty-seven of these embryos possessed a cloudy lens, 31 showed a hemorrhaged vitreous humor, and 44 had an edematous area on the neck at the back of the head. Sixty of these were smaller than normal embryos of the same age. The anomalies described did not occur in the groups supplemented with alpha-tocopheryl acetate.

Two hundred eggs were obtained from a Broad Breasted Bronze commercial turkey flock near the end of the hatching season. The turkey hens from which these eggs were obtained had been fed a good turkey breeder mash throughout the laying season. Sixty fertile eggs produced 25 poults. A high early embryonic mortality occurred. Eight of the embryos which died between the 24th and 28th day of the incubation period had a cloudy lens, were smaller in size, and were identical with embryos from dams that did not receive supplemental vit. E in the present report.

The alpha-tocopheryl acetate content of a representative sample of egg yolks from each group was determined in the laboratory of Dr. Philip L. Harris, Distillation Products Industries, Rochester, N. Y. Egg yolk from groups fed the alpha-tocopheryl acetate contained 3.6 times as much tocopheryl as those where the diet was not supplemented with this compound.

The results relative to embryonic mortality and hatchability and the influence of vit. E are similar to those obtained by Jensen(3), who stated that niacin and grass juice were not effective in reducing embryonic mortality or enhancing hatchability, but that alpha-tocopheryl acetate fed at the level used in the present experiment (20 mg/lb) was effective in increasing hatchability. No description of the embryos was given in the Jensen report (3). In the present instance supplementa-

tion of the all-vegetable protein diet with fish solubles, whey or combinations of these did not improve hatchability nor decrease embryonic mortality. The results agree also with the work of Jensen(3) that vit. E may be a more critical nutrient in turkey laying rations than heretofore considered. Further work will be necessary on the earlier stages of embryonic development in order to determine the onset and course of the anomalies described.

Summary. A cloudiness in the central portion of the lens was found in embryos from eggs laid by turkey hens fed an all-vegetable protein diet without added vit. E. Associated with the lens disorder was a hemorrhaged vitreous humor, a smaller embryo with edematous areas on the neck and feet, and a high embryonic mortality between the twenty-fourth and the twenty-eighth day of the incubation period. Supplementation of the all-vegetable protein diet with alpha-tocopheryl acetate was effective in preventing the anomalous conditions described, while fish solubles, dried whey, or combinations of these substances had no effect.

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Effect on Human Serum Lipids of Substituting Plant for Animal Fat in Diet.* (21260)

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(Introduced by V. P. Dole.)

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Several workers(1-7) have reported marked reductions of serum cholesterol levels in patients ingesting foods containing little or no fat. When fat of vegetable origin was added to such diets, a rapid rebound in cholesterol levels was observed in 3 healthy subjects by Hildreth *et al.*(8), in one hypercholesterolemic patient by Keys *et al.*(9), in 7 hypercholesterolemic patients by Wilkinson *et al.*(10), and in one normal subject by Page(11). It has been concluded from such studies that serum cholesterol levels are controlled by fat intake, regardless of source, and not by the intake of cholesterol itself(7,11). Evidence to the contrary has been reported by Kinsell and co-workers(12-15), who have shown striking reductions in serum cholesterol and phospholipid levels in patients fed diets high in vegetable fat (60-100% of calories). Simultaneously, Groen *et al.*(16) in a 9-month out-patient study of 60 normal human volunteers showed that total cholesterol levels fell slowly but significantly when vegetable fat was substituted for animal fat. A statistical study by Hardinge and Stare(17) demonstrated that strict vegetarians have lower cholesterol levels than those vegetarians who eat dairy products, and that both groups have lower cholesterol levels than the general population. Dogs have recently been reported by Tsai *et al.*(18) to have lower serum cholesterol levels on a vegetable fat diet than on a commercial diet containing variable amounts of animal fat and cholesterol.

An experiment was designed to answer the question raised by the conflicting reports cited above: Does the substitution of plant for animal fat in isocaloric amounts lead to a significant change in the concentrations of serum lipids of human subjects? The results of a 4-month study of 6 subjects are presented.

Procedure. Subjects. The age and sex of

the 6 subjects are listed in Table I. All patients were normal except for obesity of exogenous origin. Each patient's weight was held constant throughout the experiment in order to avoid effects of weight gain(19) or loss(20) on the concentration of the serum lipids. Patient 3 had had a mid-thigh amputation 15 years previously because of post-operative thrombosis after removal of an ovarian cyst. Patients 2 and 4 were Negroes, the others whites. All patients were hospitalized on a metabolic floor throughout their study; their activity was not restricted. They were permitted to leave the hospital for a few hours once per month. **Dietary regimen.** All patients were fed solid foods supplemented by fat- and protein-rich formulas. The solids permitted throughout the study included fruits, vegetables, spaghetti, macaroni, jams, jellies, and hard candies. Patient 1, for example, who consumed a total of 2775 cal. per day, derived about 1000 cal. (23 g protein, 11 g fat, 224 g carbohydrate) from solids. A certain freedom was permitted in the choice and quantity of these foods, provided enough was eaten to maintain constant weight. Table I summarizes the weight changes and dietary intakes of the 6 subjects. **Supplements** were fixed in composition and contained the major part of the protein and of the various fats under test. We insisted that the entire supplement be eaten each day. **Patients 1-4.** During the control period of animal fat feeding the supplement contained butter, salad oil, eggs, and a blended formula of milk, cream, and frozen egg yolk. During the test period of plant fat feeding the supplement contained milk-free oleomargarine (Mar-Parv), salad oil, peanut butter, avocado pear, and a blended formula of milk protein (Lesofac[†]), dextrose

* The authors wish to acknowledge the technical assistance of Miss Barbara Polancer, Mrs. Helen Richards, and Miss Margaret Hildebrandt.

[†] Lesofac (Wyeth), manufacturer's analysis as follows: Protein 50.0%, carbohydrate 39.2%, fat 1.0%, cholesterol 25 mg%, ash 5.8% (including sodium 0.02, calcium 0.80, potassium 0.85, magnesium 0.10), moisture 4.0%, vitamin B₁ 2 mg%, vitamin B₂ mg%, niacinamide 20 mg%.

TABLE I. Variations in Body Weight and in Food Intake (Means \pm S. D.) of 6 Human Subjects when Animal and Plant Food Intakes Were Altered Isocalorically, Other Dietary Intakes Remaining the Same. Proportion of total intake fed as an obligatory supplement listed separately. Differences in intakes between animal and plant regimens are not significant.

Patient	Fat feeding regimen	Duration, days†	Wt, kg	Total food intake					% of total caloric intake				Intake as obligatory supplement			
				Cal./day	Fat, g/day	Protein, g/day	Carbohydrate, g/day		Fat	Protein	Carbohydrate		Cal./day	Fat, g/day	Protein, g/day	Carbohydrate, g/day
1. ♀, 45 yr	a*	28	111.8 \pm .38	2777 \pm 80	150 \pm 2	80 \pm 2	286 \pm 36		49	12	40		1782	138	57	74
	p	70	111.4 \pm .62	2871 \pm 166	151 \pm 2	85 \pm 3	301 \pm 27		47	12	41		1771	141	61	66
2. ♀, 33 yr	a	21	100.7 \pm .25	3077 \pm 115	151 \pm 5	81 \pm 4	346 \pm 21		44	11	45		1782	138	57	74
	p	70	101.5 \pm .34	3029 \pm 160	152 \pm 4	85 \pm 4	332 \pm 37		45	11	44		1771	141	61	66
3. ♀, 47 yr	a	25	78.5 \pm .40	—	—	—	—		—	—	—		1782	138	57	74
	p†	75	79.1 \pm .58	2629 \pm 108	151 \pm 5	82 \pm 2	243 \pm 21		52	13	36		1771	141	61	66
4. ♀, 26 yr	a	35	124.0 \pm .38	—	—	—	—		—	—	—		1782	138	57	74
	p‡	49	123.9 \pm .40	3036 \pm 114	154 \pm 6	84 \pm 4	330 \pm 26		46	11	43		1771	141	61	66
5. ♀, 22 yr	a	21	122.9 \pm .16	2809 \pm 142	151 \pm 4	77 \pm 2	298 \pm 47		48	11	41		1782	138	57	74
	a	36	96.2 \pm .39	2763 \pm 210	153 \pm 6	75 \pm 8	268 \pm 48		50	11	39		1773	140	59	65
6. ♂, 16 yr	p	21	97.2 \pm .25	2577 \pm 161	151 \pm 3	75 \pm 4	230 \pm 36		53	12	36		1761	140	60	64
	a	21	97.6 \pm .45	2650 \pm 127	151 \pm 3	74 \pm 3	245 \pm 28		51	11	38		1773	140	59	65
6. ♂, 16 yr	p	21	97.8 \pm .30	2533 \pm 112	152 \pm 2	76 \pm 2	224 \pm 14		54	12	34		1761	140	60	64
	a	30	114.7 \pm .39	3983 \pm 264	206 \pm 13	105 \pm 6	428 \pm 58		47	11	43		2350	192	74	79
6. ♂, 16 yr	p	21	114.7 \pm .28	4140 \pm 83	206 \pm 6	101 \pm 4	471 \pm 30		45	10	45		2410	189	70	106
	a	21	114.3 \pm .33	4048 \pm 40	207 \pm 3	103 \pm 3	426 \pm 21		46	10	44		2350	192	74	79

* a = animal; p = plant.

† Exclusive of acclimatization and transition periods.

‡ Diet calculated only during last 41 days of plant fat regimen.

§ Diet calculated only during last 22 days of plant fat regimen.

corn oil (Mazola[†]), and milk-free chocolate syrup. The total calories and caloric composition of the 2 supplements were almost identical (Table I). In the period of animal fat feeding 140 g of fat was of animal origin and 10 g was from plant sources; in the period of plant feeding all fat was of plant origin except for traces of animal fats (<1 g/day) in the milk protein product, Lesofac. The cholesterol intakes in the 2 periods, estimated from Lange's tables(21), were 1800 mg and 23 mg/day, respectively. *Patients 5 and 6.* The only solids were butter or oleomargarine; formulas differed only in the source of fat. The animal fat supplement consisted of butter and a blended formula of milk protein (Lesofac), frozen egg yolk, cream and chocolate syrup. The plant fat supplement contained milk-free oleomargarine and a blended formula of milk protein (Lesofac), dextrose, corn oil, and chocolate syrup. Patient 6, who required more calories than the other patients, received larger supplements (Table I). The cholesterol intakes of patients 5 and 6 were 1023 and 1457 mg/day in the animal fat period, 29 and 34 mg/day in the plant fat period, respectively. The supplements fed to Patients 5 and 6, which were mainly formula feedings, appeared to affect the serum lipids in the same manner as the earlier supplements fed to Patients 1-4 which contained mixed natural foods as well as formulas. All patients except 3 and 4 were fed throughout their study by trained dietitians in a metabolic kitchen. Rejected food was weighed and subtracted in calculations of daily intake from standard tables. Patients 3 and 4 were fed for the first 8-9 weeks by the general hospital kitchen, after which time it became possible to supervise their diets in the metabolic kitchen. The foods and supplements offered these 2 patients were as similar as possible to the diets served by the metabolic kitchen, and the patients were instructed

to eat only enough of the solid foods to maintain constant weight. Their weight changes and serum lipid changes were similar in kind and degrees to those of the patients fed entirely by the metabolic kitchen. All patients were given added vitamins and ferrous sulfate daily, Unicaps (Upjohn), 2/day, and Feosol (Smith, Kline and French), 0.2 g/day. They were weighed daily before breakfast with a precision of ± 0.1 k. Fluid intake and output were measured daily. Bleedings of 35 ml were made once or twice weekly before breakfast. Two ml of blood was collected in tubes with EDTA (Versene) for hematological studies, and the remainder in plain tubes. Separate aliquots of serum were stored at 4°C and -15°C.

Methods. Total lipids were measured microgravimetrically on alcohol-ether (3:1) extracts of 2 ml of Zn(OH)₂-precipitated serum, using the Somogyi reagents(22). The same extract was used for measurements of cholesterol fractions by the Sperry-Webb method(23), and for analysis of lipid phosphorus. All measurements were made in duplicate on single extracts of serum. The

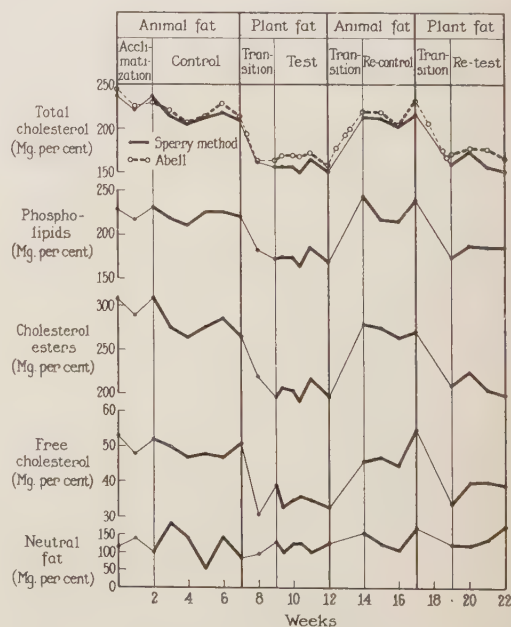


FIG. 1. Changes in concentrations of serum lipids in Patient 5 when animal and plant fat intakes were alternated isocalorically, other dietary intakes remaining the same.

[†] Mazola (Corn Products Refining Co.), manufacturer's analysis as follows: Glycerides 98.1%, non-saponifiable material 1.9%, free fatty acids 0.03%, phospholipids—trace; iodine number 125; component fatty acids—linoleic 56.2%, oleic 30.1%, palmitic 9.9%, stearic 2.9%, hexadecenoic 0.5%, myristic 0.2%, above C₁₈ 0.2%; component glycerides—mono-oleo-dilinolein 49.2%, mono-saturated-dilinolein 34.2%.

TABLE II. Concentration of Serum Lipids (Mean \pm S. D.) as Affected by Diets Containing Isocaloric Amounts of Animal or Plant Fats, Other Dietary Intakes Remaining the Same.

Patient	Fat feeding regimen	No. of weekly determinations	Total cholesterol (Sperry)		Total Ch* (Abell)		Free Ch (Sperry) μ g/100 ml serum		Ch esters (Sperry)		Total phospholipids		Neutral fat		Free Ch		Total Ch		Phospholipids $\times 100$		Free Ch		Phospholipids	
			Total		Total Ch*		Free Ch		Ch esters		Total phospholipids		Neutral fat		Free Ch		Total Ch		Phospholipids $\times 100$		Free Ch		Phospholipids	
1	a†	5	230 \pm 7.2	242 \pm 5.2	242 \pm 5.2	52 \pm 2.4	297 \pm 10.2	297 \pm 10.2	237 \pm 9.0	141 \pm 22.9	22.6 \pm 0.8	97 \pm 1.6	22.1 \pm 1.4											
	p	11	177 \pm 10.8	188 \pm 8.2	188 \pm 8.2	41 \pm 3.6	226 \pm 14.7	226 \pm 14.7	194 \pm 9.9	169 \pm 29.3	23.4 \pm 1.5	91 \pm 0.5	21.3 \pm 2.0											
2	a	4	70 \pm 1.7	80 \pm 2.3	80 \pm 2.3				117 \pm 5.7	122 \pm 7.9		60 \pm 3.7												
	p	11	52 \pm 5.8	61 \pm 2.4	61 \pm 2.4				93 \pm 4.1	100 \pm 14.5		56 \pm 5.1												
3	a	4	224 \pm 6.4	222 \pm 7.9	222 \pm 7.9	56 \pm 4.7	276 \pm 9.3	276 \pm 9.3	230 \pm 5.0	197 \pm 24.7	25.2 \pm 1.4	97 \pm 2.2	24.4 \pm 1.6											
	p	12	163 \pm 9.2	170 \pm 7.6	170 \pm 7.6	37 \pm 4.2	211 \pm 12.7	211 \pm 12.7	186 \pm 7.6	187 \pm 25.1	22.4 \pm 2.1	88 \pm 1.4	19.6 \pm 1.9											
4	a	6	208 \pm 7.8	219 \pm 8.6	219 \pm 8.6	44 \pm 3.8	274 \pm 9.3	274 \pm 9.3	203 \pm 9.2	198 \pm 19.1	21.2 \pm 1.3	101 \pm 5.5	21.8 \pm 2.0											
	p	8	172 \pm 8.1	182 \pm 5.8	182 \pm 5.8	41 \pm 3.0	217 \pm 11.4	217 \pm 11.4	186 \pm 5.9	190 \pm 26.2	23.6 \pm 1.2	92 \pm 4.8	21.8 \pm 1.6											
5	a	4	205 \pm 4.0	212 \pm 3.8	212 \pm 3.8	47 \pm 1.0	264 \pm 6.5	264 \pm 6.5	210 \pm 3.3	182 \pm 11.9	22.9 \pm 0.6	98 \pm 2.5	22.4 \pm 0.2											
	p	6	216 \pm 11.1	219 \pm 9.4	219 \pm 9.4	49 \pm 2.2	279 \pm 16.7	279 \pm 16.7	222 \pm 6.9	119 \pm 40.0	22.7 \pm 1.0	97 \pm 2.0	22.1 \pm 1.0											
6	a	4	156 \pm 5.2	168 \pm 5.2	168 \pm 5.2	35 \pm 2.2	202 \pm 9.0	202 \pm 9.0	174 \pm 7.0	120 \pm 13.7	22.4 \pm 1.5	90 \pm 1.1	20.2 \pm 1.6											
	p	4	211 \pm 5.9	219 \pm 11.1	219 \pm 11.1	48 \pm 4.6	272 \pm 6.4	272 \pm 6.4	229 \pm 14.9	140 \pm 28.6	22.7 \pm 1.6	93 \pm 4.5	21.0 \pm 1.7											
6	a	5	161 \pm 9.9	174 \pm 5.3	174 \pm 5.3	38 \pm 2.9	205 \pm 16.2	205 \pm 16.2	184 \pm 6.1	139 \pm 25.1	23.8 \pm 2.1	88 \pm 5.6	20.8 \pm 1.0											
	p	4	194 \pm 11.9	203 \pm 10.4	203 \pm 10.4	46 \pm 5.0	247 \pm 12.2	247 \pm 12.2	203 \pm 2.1	199 \pm 27.3	23.7 \pm 1.4	96 \pm 5.2	22.8 \pm 2.3											
6	a	4	145 \pm 8.1	157 \pm 8.9	157 \pm 8.9	35 \pm 3.8	185 \pm 10.1	185 \pm 10.1	161 \pm 7.5	191 \pm 20.0	23.6 \pm 1.9	90 \pm 2.8	21.5 \pm 2.2											
	p	4	197 \pm 6.9	198 \pm 4.9	198 \pm 4.9	47 \pm 2.2	251 \pm 10.6	251 \pm 10.6	193 \pm 6.4	194 \pm 27.4	23.9 \pm 1.2	102 \pm 4.4	24.6 \pm 1.8											

* Ch = cholesterol. † a = animal; p = plant.

cholesterol ester fraction was calculated as $1.67 \times (\text{total} - \text{free cholesterol})$, on the assumption that cholesterol oleate is the typical ester. Lipid phosphorus was measured by a modification of the Stewart and Hendry method(24); values were converted to total phospholipids by multiplying by the factor of 25. Neutral fat was calculated as the difference between total lipids and the sum of (free cholesterol + cholesterol esters + total phospholipids). Total cholesterol was measured independently in the same sera by the method of Abell *et al.*(25). All reported data were determined on sera stored at 4°C less than one month.

Results. The hospital study of Patients 1-3 can be divided into 4 periods: acclimatization (2 weeks), control (3-6 weeks), transition (2 weeks), and test (10-11 weeks). Patients 4-6 were studied in the same manner except that their test periods were of 3-7 weeks duration, followed by a transition period of 2 weeks and a re-control period of 3-4 weeks; Patient 5 had a third transition period of 2 weeks and a second test period of 3 weeks. In the acclimatization and control periods the dietary fat was 93% of animal origin; in test periods the dietary fat was >99% of plant origin. A feeding plan is shown in Fig. 1; the duration of the experimental periods of all patients is given in Table I.

During acclimatization, patients' weights varied until maintenance caloric intakes were established. During this period there were significant rises or falls in serum lipids in 4 of 6 patients. These data reflect variations in caloric intake and also differences between home and hospital diets; they have been omitted from this report. Data collected during the 2-week transitional periods are also omitted. Data accumulated during control and test periods are summarized in Table II.

After acclimatization was accomplished, the concentrations of the various lipid fractions of the serum varied significantly only when the dietary fat was changed from one type to the other. Once a new level was reached, there was remarkably little fluctuation from week to week. A measure of this constancy is given by the standard deviations of the mean

TABLE III. Percentage Deviations of Mean Test from Mean Control Levels of Serum Lipids—Statistical Significance of Variations.

Patient	Total cholesterol		Free cholesterol	Cholesterol esters	Phospho-lipids	Neutral fat	F/T	TC/PL	FC/PL
	Sperry-Webb	Abell, <i>et al.</i>							
1	-23%†	-22%†	-17%†	-24%†	-18%†	+20%	+4%	-6%	-4%
2	-26†	-24†			-21†	-22†		-7	
3	-27†	-23†	-34†	-24†	-19†	-5	-11*	-9†	-20†
4	-17†	-17†	-7†	-21†	-8†	-4	+9†	-9†	0
5	-26†	-22†	-25†	-27†	-21†	0	+2	-6†	-6*
6	-25†	-23†	-24†	-25†	-21†	-4	0	-8†	-9*

* $p = .01-.05$.† $p = <.01$.

F/T = Ratio of free to total cholesterol. TC/PL = Ratio of total cholesterol to phospholipids. FC/PL = Ratio of free cholesterol to phospholipids.

values for each period (Table II). To illustrate the changes found when one type of dietary fat was substituted for the other, the serum lipid concentrations of Patient 5 are shown in Fig. 1. It is seen that cholesterol and phospholipid fractions fell abruptly when plant fat was substituted isocalorically for animal fat. These values returned to their original levels when the patient was placed again on animal fat, and fell to the previous low levels when plant fat was fed a second time. Patients 1-3 were maintained on the plant fat regimen for 10-11 weeks without interruption; the low levels of cholesterol and phospholipids reached at 2 weeks on this regimen showed no escape as long as plant fats were fed. Patients 4 and 6 were returned to the animal fat regimen after 7 and 5 weeks, respectively, on plant fats; their lipid levels during the re-control period were almost identical to those of the initial control period.

Percentage deviations of mean test from mean control levels are shown in Table III. Changes which were considered significant ($p = 0.01$ or less) and which occurred in more than half the patients may be listed: 1) levels of free and esterified cholesterol and of phospholipids were lower during plant fat feeding in 6 of 6 subjects; 2) total cholesterol/phospholipid ratios were lower in 4 of 6 subjects during the plant fat regimen.

The clinical status of Patient 2, a negro female in whom there was an unexplained hypolipemia, deserves special comment. She was in excellent general health. There was no clinical evidence of hyperthyroidism; basal metabolic rate and radioactive iodine uptake were normal. Although she had had pelvic inflammatory disease many years before and

showed residual tenderness and some swelling of the right Fallopian tube, there was neither leucocytosis, elevation of sedimentation rate, nor elevation of gamma globulin at any time during her study period. Substitution of plant fat for animal fat resulted in a decrease in total cholesterol concentration from 70 to 52 mg per 100 ml serum (averaged Sperry-Webb values), and a fall in phospholipids from 117 to 93 mg. These differences were significant at a level of $p = <0.01$. Because of technical difficulties presented by its small concentration, free cholesterol was determined on only 2 sera during the control period and 2 during the test period. In the animal fat period, free cholesterol of 13.8 and 15.2 mg per 100 ml serum were found, in the plant fat period, 10.8 and 12.3 mg, with mean free/total cholesterol ratios of 21.7 and 25.4%, respectively. Calculations of neutral fat values recorded in Table II were based on the mean of these figures.

All patients felt well throughout their study. During the plant fat period there were no symptoms of increased irritability, inability to concentrate, or lack of vigor or endurance, as described by Groen *et al.* (16). Most patients passed larger stools during plant fat feeding, but there was no steatorrhea on microscopic examination. Patient 1 developed a peridental abscess in the 5th week of her test period with a temporary rise in all lipid levels; penicillin therapy and tooth extraction were followed by a return of values to the test period baseline. With this exception there were no illnesses during the study.

Discussion. Results of other studies (6, 8-11) have given rise to the widely held belief that the ingestion of fat of any type causes a

rise in serum cholesterol levels. However, in each of these experiments a test period of fat feeding was preceded by one in which little or no fat was fed. A fat-free diet imposes an unusual demand upon the body: unless the subject burns his own fat, all newly-formed lipids must be synthesized from exogenous protein and carbohydrate. Under these conditions lipids are synthesized more slowly (or utilized more rapidly), and concentrations of serum lipids fall. When exogenous fat of plant or animal origin is again made available, a rise in concentration of serum lipids can be expected to occur until a new equilibrium state is reached. Very few observations have been made in human subjects maintaining weight during prolonged periods of complete fat deprivation; these have indicated that unsaturated fatty acids may be essential dietary components for human beings (26,27). Watkin *et al.* (3) cited observations which suggested that such a deficiency may occur in some patients on Kempner's rice diet.

In contrast to those experiments in which diets containing more or less fat were tested, the present experiment aimed at comparing the effect of 2 types of fat fed at isocaloric levels, other nutrients remaining unchanged. The proportion of fat in our diets was 45-52% of calories, which contrasts with an average American intake of 40% (7). Our results in 6 subjects whose only known abnormality was obesity of exogenous origin confirm the findings of Kinsell *et al.* (12-15), who fed much larger proportions of fat to patients with various metabolic disorders. Our findings differ in 3 respects from theirs. Our data show that 1) free and total cholesterol levels decrease proportionately when plant fat replaces animal fat in the diet; 2) cholesterol levels decrease more than phospholipids; and 3) neutral fat levels are unaffected by the dietary change. The mechanism of the dietary effect and its practical value in the management of hypercholesterolemic disorders remain subjects for future investigation.

Summary. Six subjects with obesity of exogenous origin showed significant reductions in serum concentrations of free and esterified cholesterol and of phospholipids when plant fats were substituted isocalorically for animal

fats during a 4-month metabolic ward study. The approximate magnitude of the change was a 20% decrease. Neutral fat levels showed no significant change. Body weights and caloric intakes were held constant throughout the experiment.

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Technic for Complete Pancreatectomy in the Rat. (21261)

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In the rat the pancreas is a diffuse, non-capsulated, discontinuous organ lying in the gastro-duodenal and the gastro-splenic mesenteries. The bile duct for a considerable portion of its length is embedded in the pancreatic tissue. The size of the animal, the anatomical distribution of the pancreas, as well as its relation to the bile duct have been obstacles to complete removal of the pancreas. Several technics for partial pancreatectomy in rats have been published. The procedures of Ingle and Griffith(1) and of Foglia (2) have been widely used experimentally. In rats subjected to partial pancreatectomy the diabetes is of a relatively mild type (unless the animal is subjected to additional stress) and requires weeks or months to develop.

In studies in this laboratory on the origin and function of serum amylase, it became desirable to make observations on completely depancreatized rats. These are reported in a following paper(3). The procedure of Foglia(2) for 95% pancreatectomy had been employed previously and because of this experience it was decided to attempt to remove the additional 5% of pancreas which lies in the narrow band of mesentery between the bile duct and upper duodenum. By using adult animals, introducing some modifications in procedure, and with standardization of the pre- and post-operative care, a high percentage (70 to 90%) of survival of completely depancreatized rats could be obtained. The present communication describes the operative procedure and the diabetic state which ensues. Data which show that the gland is essentially completely removed are also included.

Materials and methods. Several strains of white rats, male and female, were used. Body weight ranged from 150 to 400 g. The animals were kept in individual metabolism cages and received the synthetic low-residue diet described below. Qualitative tests for glucose and acetone bodies were run on 24 hour urine samples. Glucose tolerance was determined as follows: after an overnight fast, an 0.1 ml sample of tail blood was taken and 3.5 g of glucose per kg of body weight injected intraperitoneally as a 10% solution. Subsequent blood samples were taken at $\frac{1}{2}$, 1, 2, 3, and 5 hours. Blood sugar was determined by a combination of the methods of Somogyi(4) and Nelson(5). Tissue amylase was determined by a modification of the method of Smith and Roe(6). The low-residue diet had the following composition: 30% casein, 25% lard, 14% starch, 14% sucrose, 5% salt mixture, 10% brewer's yeast, and 2% cod liver oil.

Operative procedure. The rats were placed on the low-residue diet at least 3 days prior to operation and fasted 18 to 24 hours immediately preceding it. The low-residue diet and the fasting period insured absence of solid material in the stomach and intestine. If there was an appreciable amount of residue in the stomach or intestine there was considerably more hemorrhage during and after operation. The animal is given an intraperitoneal injection of 0.05 ml of nembutal (60 mg/ml)/100 g body weight. The abdomen is moistened with 70% alcohol and opened by a mid-line incision extending about 3.5 cm caudal-ward from the xiphoid process. The stomach is drawn out and turned upward so that the

anterior surface lies on the thorax. This movement usually brings out the spleen. If not, it is exposed by drawing out the mesentery between it and the stomach. The gastrosplenic mesentery contains about $\frac{1}{2}$ of the pancreas. The transverse colon is pushed in a caudal direction. This exposes the origin of the pancreatic and splenic blood vessels and the attachment of the mesentery to the posterior abdominal wall. Exposed organs and the fingers of the operator are kept moist with sterile saline. Small swabs made by twisting a small piece of cotton about the end of a toothpick are employed for removal of the pancreas. The mesentery is held on the ball of the index finger and the pancreas rolled out of the mesentery in small pieces by gentle rubbing of the exposed moist surface. Removal of the pancreas in this area is begun by rubbing upwards and outwards on the mesentery from its point of attachment to the posterior wall of the abdomen. This is continued upward to the spleen and centrally to the greater curvature of the stomach. Care must be taken to preserve the splenic blood vessels. However, the rubbing may be done directly on the vessels as they rest on the index finger without danger of serious hemorrhage. Particular attention is paid to complete removal of pancreatic tissue about the plexus of vessels leading into the hilum of the spleen and that immediately adjacent to the stomach. Rubbing is carried downward along the greater curvature of the stomach to the pyloric end with particular attention to the gastro-duodenal and gastro-epiploic arteries. To insure complete removal of all pancreatic tissue most of the fat is also rubbed out so that all that remains are the larger blood vessels and fragments of mesentery. Small blood vessels leading into the pancreatic tissue may be disrupted without severe hemorrhage. The spleen is now returned to its normal position in the abdomen. The first loop of the small intestine is then exposed by exerting traction on the duodenum at its origin from the stomach. The remainder of the pancreas is located in the mesentery within this duodenal loop of intestine. After the loop has been exposed the rat is rotated so that its head points to the operator's right. By gentle pressure with the aid of a swab the loop is

freed from its attachment to the transverse colon. Considerable care must be taken here because during the separation the portal vein is exposed and may be easily ruptured. At this point the course of the bile duct should be determined. By holding up the loop of duodenum the bile duct may be seen appearing near the pylorus of the stomach and passing obliquely through the mesentery until it enters the duodenum at about $\frac{1}{3}$ the length of the loop from the stomach. The 5% of the pancreas situated between the bile duct and duodenum is removed first. This is done with gentle rubbing, starting at the point of attachment to the stomach and working downward and parallel to the bile duct. Then the pancreas is removed from the rest of the mesentery within the loop, starting along the line of the portal vein and working upward and outward. Usually all of the pancreas can not be conveniently removed while working from only one side of this mesentery, so after the pancreas on one side has been removed the position of the rat is reversed and the opposite side of the mesentery is rubbed to remove the remainder of the pancreas. Care must be taken to remove all pancreas which lies against the inner curvature of the duodenal loop. The organs are replaced and the animal closed in 2 steps. Peritoneal and muscle walls are closed together with a continuous suture. The cutaneous layer is closed with an interrupted stitch or with metal skin clamps. Immediately after the incision is closed, the rat is given two 4 ml injections of isotonic saline subcutaneously in the dorsal region and 0.01 ml of penicillin (300000 units/ml) subcutaneously in the lateral aspect of the thigh. Twenty to 30 minutes are required for the entire procedure. Some small blood vessels are ruptured or destroyed during the operation. However, at autopsy in animals surviving for several days or longer there has been no evidence of failure in blood supply to any organs. While it is important to avoid damage to the bile duct this structure can withstand considerable gentle rubbing with a swab if supported on the index finger. For the most rapid removal of the pancreas the surface should be kept moist with saline and dry swabs used. We use 20-30 swabs/operation.

Following operation the animal is kept at

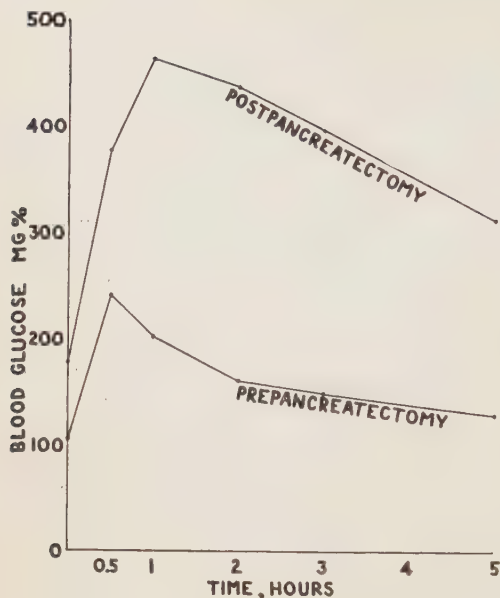


FIG. 1. Glucose tolerance curves before and after pancreatectomy. Each point, avg of 9 rats.

about 35°C (for 72 hours) and given no food or water for 24 hours. After this period the animal is given saline to drink for 48 hours. The low residue diet is started in the afternoon of the second day post-operative. The post-operative fast allows the intestine to recover from trauma caused by handling during the operation. At present we obtain 6 or 7 operative survivors out of each group of 8 rats. Deaths are due either to severe hemorrhage, usually the portal vein, in which case the animal dies within 24 hours, or to rupture of the bile duct or biliary obstruction. In the latter case death usually occurs after several days.

Post-operative observations. Rats, depancreatized by the above technic, have been observed for periods up to 90 days. The animals appear healthy, eat well, and gain in weight on either the low residue diet or a standard laboratory chow (after 7 days) without administration of insulin. In about 80% of the animals a marked protracted glucosuria appears in 1-3 days after return to the diet. The remainder exhibit an intermittent glucosuria. Polyuria (15-50 ml/24 hr) is also present. Acetonuria has not been observed in animals on the low-residue diet or laboratory chow. Fig. 1 shows the average glucose tol-

erance curves of 9 representative animals before and 12 to 22 days after pancreatectomy. All animals, regardless of degree of glucosuria, gave a typical diabetic response after pancreatectomy. Representative animals, 60 to 90 days post-operatively, were sacrificed and block sections of the area normally containing the pancreas prepared. Histological evaluation of sections from an animal, giving the typical diabetic response described above, revealed some remnants of pancreatic tissue in which the acinar cells were less compact than normal; the granules were indistinct and diffuse with a decrease in staining properties. No islet cells were found. Sections from an animal, in which the operation was considered unsatisfactory because of the mildness of the diabetes, contained remnants with essentially normal acinar cells and a few small islet cells.* In evaluating the technic for total pancreatectomy in the rat it was felt that because of the diffuse nature of the gland some procedure in addition to histological examination should be employed. The procedure adopted was to remove from representative animals all tissue in areas which the pancreas normally occupies and analyze this tissue for its amylase content. Rat pancreas has more than 1000 times as high a content of this enzyme as any other tissue (with the possible exception of salivary glands), so that the presence of any pancreatic tissue in the area would be evidenced by a relatively high amylase content. Parallel analyses on comparable tissues from unoperated animals gave a basis for calculating the percentage of the gland remaining in operated animals. Amylase levels obtained were: for 9 unoperated animals, 25,799 units with S. D. of $\pm 6,849$ and for 9 operated animals 53.7 ± 35.8 units per g of fresh tissue. Assuming that all amylase activity in this area in operated animals was due to pancreatic acinar cells, at least 99.8 of the pancreas had been removed.

Summary. A technic, giving a high survival rate, for essentially complete removal of the pancreas in rats is described. The use of a

* The sections were evaluated by F. N. Miller and I. R. Telford, George Washington University Medical School.

low residue diet and standardized pre- and post-operative care appear to be mainly responsible for the high survival rate.

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Reduction of Pain-Conditioned Anxiety by Analgesic Doses of Morphine in Rats. (21262)

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Previous investigations(1,2) have demonstrated that an analgesic dose of morphine (15 mg) reduces the behavior-disturbing effects of anticipation of pain in man. The present study was designed to test the hypothesis that morphine also reduces anxiety associated with anticipation of pain in animals. Confirmation of this hypothesis may lead to the development of an analgesic testing method in animals which differs in principle from previously reported technics(3-6).

In accordance with this objective, it was considered essential that the method meet the following criteria: a) The response must be easily quantified, b) Induced changes in the response should be based upon principles similar to those underlying the human work, and c) The response change must be sensitive to graded doses of opiates. In addition, the method should, if possible, meet a requirement proposed by Wikler(7): It should distinguish between analgesic, hypnotic, and paralytic effects of drugs. The present paper is a presentation of the methodology employed with rats and of the results obtained on a first experiment on morphine.

Methods and materials. The conditioned operant or instrumental response of bar-pressing for food rewards under food deprivation was studied in a modified Skinner Box(8). Partial or complete cessation of bar-pressing, considered to be conditioned anxiety or inhibi-

tion, was produced by pairing a tone with electric shock. The degree of restoration of bar-pressing followed subcutaneous administration of graded doses of morphine provided measures of the reduction of the inhibition. Correspondence between the drug dose and the % of restoration of the previous response rate was the pertinent datum. The subjects were 12 male albino rats approximately 4 months of age when the experiment was begun. However, the N's as shown in Table 1 for the various test days are not equal since an animal was omitted from the experiment when its bar-pressing behavior was severely impaired by the morphine injections. To produce a high and fairly constant rate of response which would survive the conditioning procedure and drug injections, the animals were maintained at approximately 70% of satiation weight; they were weighed daily and fed individual amounts of Purina laboratory chow. Water was always available in the home cage but not in the experimental apparatus. The Skinner Box was modified in several respects. It was somewhat smaller than the original design. A conditioning chamber (8 x 7 x 10 inches), the floor of which was a barred shocking grid, was mounted in a sound proof box (14 x 14 x 18 inches); the bar and other accessories were similar to those described by Skinner. Circulation of air was insured by the action of a small vacuum pump.

Observation windows were placed in one end of the apparatus, and its interior was illuminated by a small electric light bulb. Conditioned bar-pressing was developed by the Skinner method, and the animals were trained on a periodic 2-minute schedule which was later changed to a periodic reinforcement with a 2-minute mean. After the animals had acquired a rapid bar-pressing rate and produced relatively smooth curves on a cumulative recorder, the technic of Estes and Skinner(9) for developing conditioned anxiety was introduced.* Conditioned inhibition of bar-pressing was developed by sounding a 60-cycle tone for 4 minutes which was terminated by an A. C. shock delivered through the floor grid. The tone was applied through a headphone which was fastened to the top of the box. The electric shock, ranging from 40 to 60 volts for approximately 0.5 seconds duration, was administered through a variac, and was adjusted according to each animal's performance during the tone-shock conditioning. The administration of drugs was begun with adaptation periods of simple bar-pressing under 3 increasing doses of morphine. During testing procedures, control and experimental periods were 48 hours apart; control readings always being obtained first. Conditioning was omitted on the day preceding each of the recording periods. On drug administration days, each animal was weighed, injected subcutaneously, and placed in the home cage for 75 minutes to allow for approximate peak action of the drug before being placed in the Skinner Box. At intervals of 7 to 10 days morphine sulfate was injected in the following order, 8, 10, 6, 11, 4, 5, 14, 12, 11, and 13 mg/kg. Reproductions of typical morphine records are shown in Fig. 1, B and B'. Since morphine produced some decrease in the rate of response in the majority of animals, calculations for each animal were based on its performance during the 4 minutes immediately preceding the tone; cumulative bar-pressings for the 4 minutes of tone presentation were calculated as percentages of each animal's previous rate. Drug and

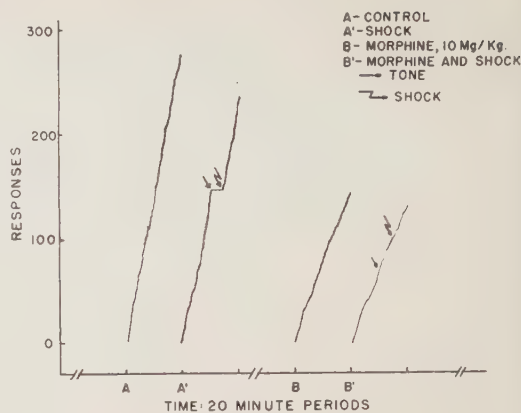


FIG. 1. 20-min. cumulative recordings. As the kymograph moved to left, responses were cumulated vertically.

non-drug records were treated in the same manner, each animal serving as its own control.

Results. A 20-minute record of a well-trained animal is shown in Fig. 1, A'. It may be observed in this typical, although individual, record that responding ceased for the 4 minutes while the tone sounded and was immediately resumed after the presentation of the shock with little, if any, compensatory increase in rate. The mean control % was 13.4, the median was 17.3, and the range was 0 to 26%. Table I presents the data in rounded percentages, and Fig. 2 shows a free-hand curve fitted to the obtained points.

Correspondence between restoration of bar-pressing and the dose level of morphine is obvious. The results as presented in Fig. 2 demonstrate a range of approximately 4 to 10 mg/kg, a factor of $2\frac{1}{2}$. The upper limit of effectiveness appears to be 80% restoration, or slightly greater, without complication by impairment of performance. As indicated in Fig. 1, discrepancies were found in the higher dose range. Although response rate was restored to a median of 80% by these higher doses, activity and bar-pressing were impaired

TABLE I. Mean Response Rate Percentages for Graded Doses of Morphine and the Number of Animals Tested.

MS	mg/kg													
	0	4	5	6	8	10	11	11	12	13	14			
%	11	22	28	37	54	80	97	80	67	82	78			
No.	—	6	5	9	5	6	6	6	7	4	2			

* Later, Hunt and Brady(10), and Brady(11), employed a similar method for investigating the effects of electro-convulsive shock and tetraethylammonium on a conditioned "emotional" response.

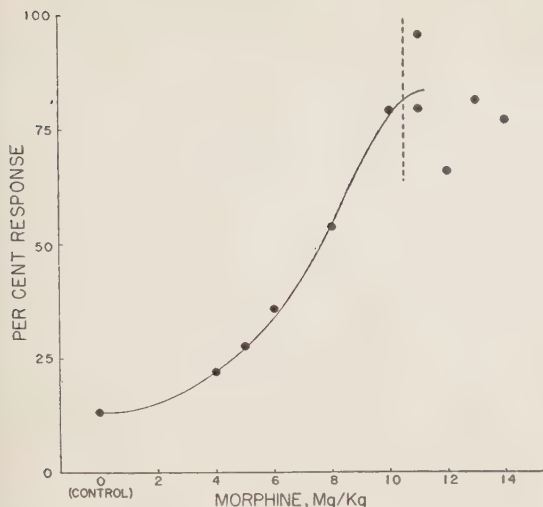


FIG. 2. Morphine dose-effect relationship. Each point on curve represents mean performance when percentage for each animal was calculated in terms of its previous rate.

at 12 mg/kg and above.

Discussion. Application of the principles derived from studies on human subjects to the behavior of the laboratory rat were found to be quite feasible. The reduction or elimination of bar-pressing during the period of the tone is considered to be the disrupting effect of anticipatory response to painful stimuli; this is analogous to the findings on man. Analogous, also, were the changes in behavior following administration of morphine; the effects of anxiety on the measured responses were either reduced or abolished. One of the chief differences between these studies was that only one dose level of morphine was employed with the human subjects whereas 13 were used with rats; in the latter the restoration of bar-pressing was proportional to the dose from 4 to 10 mg/kg.

The higher dose range, however, presents unique problems; the hypnotic and neuromuscular effects of morphine, particularly drowsiness and ataxia, are frequently marked in doses higher than 11 mg/kg. As shown in Fig. 2, a considerable degree of scatter occurred with doses of 12 mg/kg and above. This was partly due to side-effects of morphine which produced irregularities of performance including marked impairment of bar-pressing. Reliable measures of the analgesic effect of

morphine, then, would extend from the smallest dose which could be differentiated from control performance, to the highest dose which produced a relatively small standard error and a statistically significant increase of bar-pressing over that produced by an arbitrarily chosen lesser amount. Thus, the maximum dose of morphine which produced analgesia without marked impairment of normal behavior might be distinguished from those doses which produced analgesia with concomitant hypnotic and unfavorable neuromuscular effects.

Further studies are being undertaken in which improved control of variables and statistical evaluation of differences will be obtained. These studies will use a greater number of animals, improved apparatus, more constant temperature and humidity control, and possibly additional measures of inhibition. Comparative measures will be obtained for the actions of a sufficiently wide range of drugs to thoroughly test the described procedure.

Although as yet the method has not been shown to be specific for detecting analgesics, it is sensitive to graded doses of morphine, and differentiates between anxiety reduction and hypnotic and paralytic drug effects. In addition, the results, which were strikingly similar to those of the previous human work, provide strong supporting evidence for the hypothesis that one of the major actions of a potent analgesic is the reduction of behavior-disrupting anticipatory response to noxious stimuli.

Summary. 1. Rats, maintained at 70% of satiation weight, were conditioned to press a bar at a rapid and constant rate in a modified Skinner Box. After about 15 days of training when this behavior had been thoroughly established, a method for producing conditioned anxiety was introduced. Shortly after each animal began the daily bar-pressing session a 60-cycle tone, which sounded for 4 minutes, was terminated by the application of a strong electrical shock. After several days of conditioning this procedure produced almost complete cessation of bar-pressing during the tone period. In testing the effect of a known analgesic the administration of graded doses of morphine (4-11 mg/kg) produced proportional restoration of the inhibited bar-pressing.

2. The reduction or elimination of inhibition by morphine was considered to be a reduction of anxiety associated with anticipation of noxious stimuli. The results parallel in all essential details previous methodological work on man, and strongly suggest that the present procedure may be useful as a technic for the screening of possible analgesic drugs.

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Observations on G-25671, A Phenylbutazone Analogue (4-(phenylthioethyl)-1,2-diphenyl 3,5-pyrazolidinedione).^{*} (21263)

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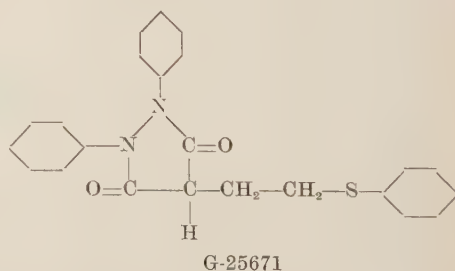
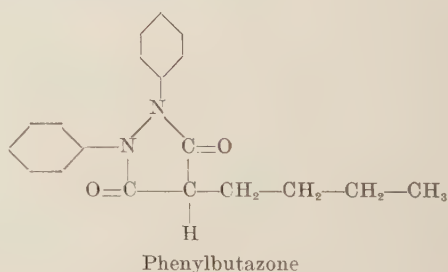
From the National Heart Institute, National Institutes of Health, Bethesda, Md., the New York University Medical Division, Goldwater Memorial Hospital; Departments of Medicine, Mount Sinai Hospital, and Columbia University College of Physicians and Surgeons, New York.

Phenylbutazone (Butazolidin) has been shown to be a potent antirheumatic agent but it may produce such side effects as edema, gastric distress, skin reactions and, on rare occasions, gastrointestinal hemorrhage and bone marrow depression. In an attempt to develop a drug retaining the antirheumatic action of phenylbutazone but devoid of its side effects, a series of phenylbutazone analogues are being screened in animals and man for anti-inflammatory activity.[†] It is the purpose of this paper to report preliminary observations in man on one of the compounds (G-25671) which showed marked anti-inflammatory effects in the animal screening tests.

^{*}Supported by grants from the Public Health Research Institute of City of New York, Josiah Macy Jr., Foundation, National Institute of Arthritis and Metabolic Diseases, and New York Chapter of Arthritis and Rheumatism Foundation.

[†]The compounds, supplied by Geigy Pharmaceuticals, were synthesized by Haefliger, F., *et al.*, and screened in animals by Domenjoz, R., and Wilhelm, G.

For comparison, the structures of phenylbutazone and G-25671 are shown:



Materials and methods. Concentration of G-25671 in plasma was determined by a

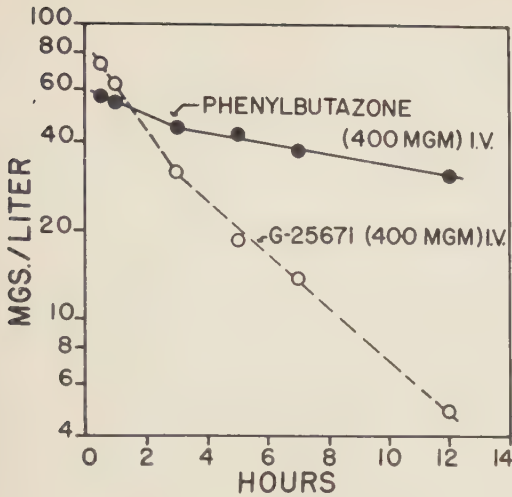


FIG. 1. Plasma levels of phenylbutazone and G-25671 after single intravenous dose of the drugs to same subject.

method essentially the same as that used for phenylbutazone(1), except that 3% isoamyl alcohol was added to the petroleum ether used for extraction of the drug and the spectrophotometric assay was made at 255 mu. The specificity of the method for the drug in plasma was established by counter-current distribution. Urinary sodium and potassium concentrations were determined with a flame photometer using lithium as an internal standard. Urinary chlorides were measured by the method of Wilson and Ball(2). Serum and urinary uric acid concentrations were estimated by a modification of the method of Buchanan, Block, and Christman(3), employing uricase, urea-cyanide-carbonate and arsenophosphotungstic acid. For investigation of the physiological disposition of the drug and its effect on electrolyte excretion, 12 non-arthritic patients without obvious heart, liver or kidney disease were used.

Results. Rate of disappearance of G-25671 from plasma. Five subjects received an intravenous injection of 400 mg of G-25671 over a 10-minute period and drug plasma levels were measured at various times thereafter. The levels declined at a rate averaging about 20%/hour (Fig. 1) after equilibrium between plasma and tissues had been achieved. Since only minimal amounts of the drug are excreted in the urine and since it is not extensively

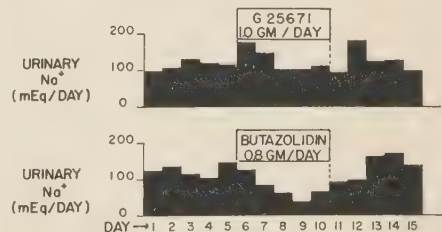
localized in tissues relative to plasma, this decline must represent its rate of biotransformation. The biotransformation of phenylbutazone, on the other hand, is much slower, averaging 20%/day (Fig. 1).

Absorption of G-25671. Information concerning the absorption of G-25671 from the gastrointestinal tract was obtained by comparing plasma levels of G-25671 in 2 subjects following oral and intravenous administration of single doses of 400 mg of the drug. Plasma levels obtained 3 hours after administration were approximately the same by either route, indicating that absorption was rapid and virtually complete.

Plasma levels on repeated dosage. Ten subjects received daily one g of G-25671 orally, in divided doses, for a period of 6 days. The drug plasma level 5 hours after the morning dose ranged from 30 to 60 mg/L. Within 24 hours after discontinuing therapy the drug was no longer detectable. By comparison, when patients received 800 mg of phenylbutazone daily, the plasma level ranged from about 80 to 160 mg/L 5 hours after discontinuing dosage and persisted in detectable quantity for a period of from 7 to 10 days.

Effect on urinary excretion of water, electrolytes and phenolsulfonphthalein (PSP). The effect of G-25671 and phenylbutazone on urinary excretion of fluid and electrolytes was

COMPARISON OF THE EFFECT OF G25671 & BUTAZOLIDIN ON URINARY SODIUM



	G25671	BUTAZOLIDIN
MAXIMUM CHANGE IN HEMATOCRIT	43→45	46→40
MAXIMUM CHANGE IN BODY WEIGHT(kg)	46.7→46.8	48.6→50.5
URINARY Cl ⁻	NO CHANGE	DECREASE SIMILAR TO Na ⁺
URINARY K ⁺	NO CHANGE	NO CHANGE

FIG. 2. Effect of G-25671 and phenylbutazone in same subject on urinary electrolytes, body wt and hematocrit.

compared in 2 subjects on a controlled sodium intake of about 100 mEq per day. No demonstrable retention of fluid or electrolytes was observed during the administration of G-25671 in daily oral doses up to 1.5 g in contrast to the marked retention of sodium produced by phenylbutazone (Fig. 2). It will be noted that G-25671 produced no weight gain or fall in hematocrit such as was observed during phenylbutazone administration. The effect of G-25671 and phenylbutazone on the excretion of PSP was compared in 3 subjects. Both G-25671 and phenylbutazone produced a significant decrease in the rate of excretion of PSP (Table I).

Effect on urinary urate excretion. The drug was found to exert a marked uricosuric effect. The mean increase in urinary uric acid excretion in 4 gouty subjects given 1 g per day by mouth (in divided doses) and maintained on a constant low purine diet was 42% in the first 24 hours of medication, 91% in 48 hours and 65% in 72 hours. The mean decrease in serum uric acid concentration in nine gouty subjects including the 4 subjects in whom the urinary uric acid excretion was measured was 18% after 24 hours, 45% after 48 hours and 49% after 72 hours. A characteristic increase in urinary urate excretion with accompanying fall in serum urate level is shown in Fig. 3. The uricosuric effect ceased rapidly upon discontinuance of medication (Fig. 3). The uricosuric action of G-25671 is more marked than that observed with phenylbutazone(4).

Anti-inflammatory effects in man. Currie has already observed that G-25671 exerts an antirheumatic effect in subjects with rheumatoid arthritis(5). Preliminary trials were

URICOSURIC EFFECT OF G25671 IN CHRONIC GOUT

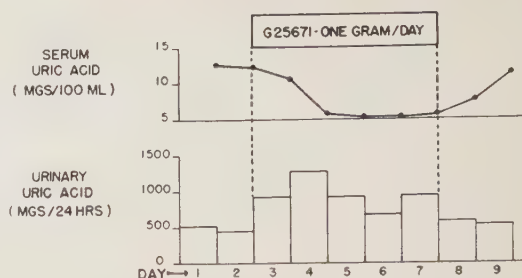


FIG. 3. Effect of G-25671 on serum and urinary uric acid in subject with chronic gout.

carried out by us in 10 patients with active rheumatoid arthritis and 10 with acute gouty arthritis. The drug was administered in doses of 1 to 1.6 g per day. A distinct anti-inflammatory effect was noted in most of the cases but the effects in general appeared to be less marked than those to be expected with phenylbutazone. The only overt side effect noted over periods of up to two weeks medication was a drug rash in one patient, which rapidly subsided on stopping the drug. Long-term toxicity studies in animals are now in progress. Should further experience indicate that the drug has such low order of toxicity that long-term administration is feasible it may have clinical application, particularly in the treatment of chronic gouty arthritis because of its combined uricosuric and anti-inflammatory properties.

Summary and Conclusions. 1. Observations were made on the physiological behavior of a compound in which the butyl side chain of phenylbutazone was replaced by a phenylthioethyl group. 2. Rate of biotransformation of this compound in man was greatly accelerated, with a biologic half life of only 3 hours compared to 70 hours for phenylbutazone. Such rapid disappearance might have some advantage in minimizing toxic manifestations but would pose the problem of maintaining therapeutic levels since the drug would have to be given at relatively frequent levels. 3. Like phenylbutazone, G-25671 exerted distinct antirheumatic effects but produced little or no sodium retention and consequently no hemodilution. These observations show that the antirheumatic and sodium-retaining properties in the phenylbutazone

TABLE I. Comparison of Effect of G-25671 and Phenylbutazone on Phenolsulfonphthalein (PSP) Excretion, in 3 Patients.

% of dose of PSP excreted in 30 min.		Effect of phenylbutazone	
Effect of G-25671		Control	After drug†
Control	After drug*	Control	After drug†
33.0	8.5	35	15.0
40.5	26.0	50	29.5
30.0	21.5	35	22.5

* 1 g orally given 3 hr before inj. of PSP.

† .8 g *Idem*

series may be dissociated. 4. The drug has a more marked uricosuric effect than phenylbutazone.

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Phospholipid Studies of Different Serum Lipoproteins Employing P₃₂. (21264)

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The distribution of the lipoproteins of human serum into 2 main types, the α and the β lipoproteins, is now well established as a result of chemical(1,2) and electrophoretic fractionation(3,4). Each of these contains phospholipid and the average normal serum shows approximately equal amounts of α and β phospholipid. The two lipoproteins differ considerably in cholesterol and protein concentration(2,3,5). A number of studies have been directed toward an investigation of phospholipid turnover employing P₃₂ both in animals and humans (6-8). These have been mainly concerned with total serum phospholipid measurements. The purpose of the present report is to present data concerning phospholipid P₃₂ analyses in the individual types of lipoproteins separated electrophoretically.

Material and Methods. Six patients, 3 normal individuals and 3 patients with mild liver disease, were employed in this study. Each was given approximately 0.2 millicuries of P₃₂ in the form of Na₂HPO₄ orally. Bleedings were usually made at 6, 12, 24 and 48 hours after administration. Electrophoretic separation of the lipoproteins was carried out in a starch supporting medium as described previously(3,9). Broad starch blocks were usually employed, measuring 22 cm in width, 45 cm in length and 1.2 cm in thickness. This permitted the separation of 3 samples at the same time furnishing a direct comparison. Samples ranging from 2-10 cc were applied to the starch block and the separation carried out

at 350V over a period of 18 hours in a cold room at 4°C. The starch block encased in wax paper was kept in a relatively dry state throughout the procedure. Following the separation, the starch block was cut up into 1/2 inch segments which were then thoroughly dried before a fan. These were then each extracted with 25 cc of alcohol-ether (Bloors reagent) and these extracts employed for phosphorus analyses and for radioactivity measurements. The alcohol-ether extracts were evaporated down to approximately 4 cc volume and transferred to planchettes for further evaporation to dryness followed by counting. Recoveries of serum lipid phosphorus in the fractions ranged from 85-102% by this procedure. An alternate procedure that was employed for purposes of verification was to obtain the aqueous protein solution directly from the undried starch segments by displacement filtration(9). The protein was then precipitated by ZnSO₄ and NaOH(10), washed, and extracted with alcohol-ether. Similar results were obtained by the two procedures although the latter was more time-consuming and recoveries were not as good as by direct extraction of the dry segments. Barbitol buffer pH 8.6, $\Gamma/2$ 0.05 was used in these experiments. Phosphorus was converted to phospholipid by the factor 25.

Results. The distribution of radioactivity in the lipid extracts of the starch segments following electrophoretic separation of normal sera taken 12, 24 and 48 hours after oral ad-

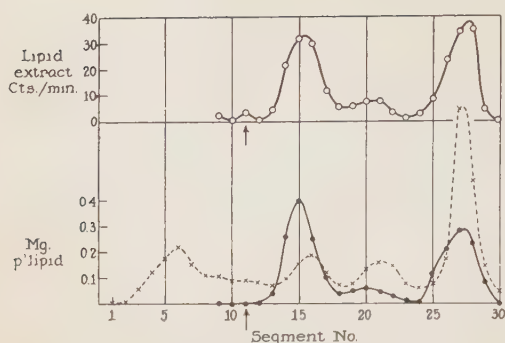


FIG. 1. Curves showing distribution of phospholipids and radioactivity in various fractions of a normal serum separated by zone electrophoresis. Upper curve illustrates radioactivity of alcohol-ether extracts; lower curve phospholipid on similar extracts. The β lipoprotein peak, segment 15; a_2 peak, segment 20; a_1 peak, segment 27. Broken line represents protein curve on the same fractions. Arrows show the site of application of serum.

ministration of P₃₂ was found to be very similar to that for total lipid phosphorus. Fig. 1 illustrates a typical experiment with a non-fasting 12 hour serum. The lower curves show the protein and phospholipid distribution obtained from analyses of the same starch segments. The protein curve shows no a_1 peak and a wide gap between the β and γ -globulin peaks. This is characteristic of the pattern obtained in this buffer of 0.05 ionic strength. Three phospholipid peaks are visible, a high β lipoprotein (segment 15), a small a_2 lipoprotein and a large a_1 lipoprotein peak. The a_2 lipoprotein is well separated from the β in this weak buffer. The separation of these 2 lipoproteins is much more difficult in $\Gamma/2$ 0.1 barbital buffer. The upper curve shows the distribution of radioactivity in the alcohol-ether extracts obtained from another aliquot of the same serum separated on the same starch block and cut into identical $\frac{1}{2}$ inch segments. Because of the low radioactivity of the serum, it was not possible to obtain this broad distribution of fractions when protein, phosphorus and radioactivity measurements were carried out on identical fractions. The curve for radioactivity closely parallels that for phosphorus suggesting that the different lipoproteins have very similar specific activities. This was also found to be the case at 24 and 48 hours. Table I shows the specific ac-

tivities for the 3 lipoprotein fractions at various time intervals. Here the counts and the phosphorus analyses were carried out on the same pooled fractions. A second sample on another portion of the starch block was used for phosphorus analyses to indicate where to cut the main portion of the block into the three lipoprotein fractions. The specific activities obtained were similar and the variations fell within the experimental error.

The a_2 lipoprotein illustrated in Fig. 1 and Table I has not been well characterized. In a previous report(3), this fraction was described as being evident in certain normal sera. Further observations have indicated that it is very low in fasting normal sera but rises after meals, particularly after fatty meals, and is associated with a large part of the turbidity of non fasting sera. In 5 normal individuals where this fraction was specifically studied, it was found to be very low in 4 of the 5 individuals after fasting for 15 hours. In all cases it rose after a meal and disappeared rapidly after heparin injections. This fraction usually contained slightly more phospholipid than cholesterol but the cholesterol-phospholipid ratio was not found as low as in the a_1 lipoprotein. In the radioactivity experiments where blood was drawn at variable times after the patient had eaten, the a_2 lipoprotein phospholipid varied considerably in concentration. In Table I the levels at 7½ hours and 10½ hours in non-fasting sera were considerably higher than the level in the fasting 24 hour serum. The specific activity of this fraction, consisting at least in part of post prandial phospholipid was not very different from the other lipoprotein fractions, although

TABLE I.

Hr		Phospho- lipid, mg	Ct/min.	Sp. act., ct/mg
7½	β	.69	36	52
	a_2	.27	16	59
	a_1	.42	24	57
10½	β	.62	74	120 123*
	a_2	.31	46	150 152*
	a_1	.51	64	126 145*
24 (fast- ing)	β	.53	75	140
	a_2	.08	9	110
	a_1	.47	63	135

* Separate experiment at 10½ hr.

the relatively low concentration of this component made this assay less accurate than for the other lipoproteins.

In certain experiments where sera at early time intervals were separated immediately after removal from the patients some difference in specific activity was noted. The α_1 lipoprotein appeared to have a slightly higher specific activity than the β . This difference seemed to decrease in the same serum with time even when it was stored at 4°C. Special care was employed in these experiments to avoid contamination by other phosphorus containing compounds that show similar mobility to these lipoproteins. The phospholipids were subjected to repeated extractions from the dry state with different solvents. Similar results were obtained and the α_1 lipoprotein contained higher counts relative to phosphorus than the β although the difference was not great. This difference was noted in the sera of certain individuals; others showed identical specific activities for the two lipoproteins at each time interval. In no experiments was a higher β lipoprotein specific activity encountered; the specific activities were either equal or slightly higher in the α_1 fraction.

A number of phosphorus compounds other than phospholipid that separated in the electrophoretic experiments were encountered. The starch medium proved less suitable for the localization and identification of these substances than for the separation of the lipoproteins. Better resolution of the former was obtained in a paper pulp system(9) and the results of these experiments will be described separately.

Results *in vitro*. In view of the similarity of specific activities of the lipoprotein fractions at various time intervals following oral administration of P₃₂ and in view of the suggestive evidence for further equilibration on standing, a series of *in vitro* experiments were carried out. The α_1 and β lipoproteins were isolated from sera 24 hours after P₃₂ administration. Great care was taken not to include any of the other lipoprotein in these isolations and the α_2 and overlapping fractions were discarded. The purity of the α_1 fractions and the β fractions was tested by electrophoretic sepa-

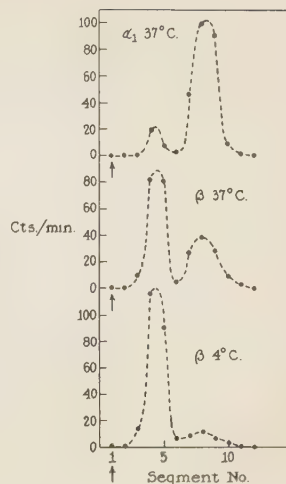


FIG. 2. *In vitro* shift in radioactivity from one lipoprotein to another. Upper curve: distribution of radioactivity of lipid extract of electrophoretic segments following 2 hr incubation of labelled α_1 lipoprotein (segments 7, 8, 9) and unlabelled β lipoprotein (segments 4, 5). Lower curves: 2 hr incubation of labelled β lipoprotein with unlabelled α_1 lipoprotein.

ration and in each case no β was contaminated with α_1 or the reverse. The mobility of the isolated β lipoprotein fractions were noted to be slightly more rapid than in the original serum but good preparations still migrated considerably slower than the α_1 lipoproteins and could be distinguished.

Following incubation of these isolated labelled lipoprotein fractions either with whole serum or with non labelled isolated fractions, a transfer of radioactivity was noted. Fig. 2 illustrates a typical experiment showing the results obtained on mixing equal amounts, in terms of phospholipid, of the labelled and unlabelled lipoprotein fractions. The incubation period in this experiment was 2 hours duration and the 3 samples were separated electrophoretically on the same starch block. The lower two curves show the shift of radioactivity from the β lipoprotein to the α_1 lipoprotein at 4°C. and at 37°C. A considerably greater transfer occurred at the higher temperature. The upper curve illustrates the shift of radioactivity from the α_1 lipoprotein to the β lipoprotein at 37°C. A definite shift occurred but this was not quite as great as in the reverse direction. Determinations of lipid phosphorus as well as

radioactivity demonstrated that the transfer of radioactivity was an exchange reaction. There was no detectable shift in phosphorus after incubation. The specific activities of the two lipoproteins approached equality in some experiments in which the incubation period was 4 hours or more; the fraction that was labelled to begin with, however, usually showed a slightly higher specific activity than the receptor fraction.

Following heating of the labelled and unlabelled fractions to 60°C. for 15 minutes prior to mixing and incubation, the transfer of radioactivity was markedly reduced. The lipoproteins did not become turbid in these experiments and their mobility was close to that observed with the unheated fractions. Potassium cyanide and potassium fluoride, when added prior to incubation did not inhibit the exchange. Addition of alcohol extracts containing labelled phospholipid did not result in significant transfer of radioactivity to the lipoproteins after incubation. In these experiments the radioactivity remained at the site of application and did not migrate with the lipoproteins. The latter experiments were not completely satisfactory technically because of the difficulty of adding the phospholipid to the aqueous lipoprotein solution. Studies of the *in vitro* exchange of P₃₂ phospholipid in the α_2 lipoprotein fraction were not entirely satisfactory because of the relatively small amount of material and greater difficulty in isolation without contamination. However, the results suggested an exchange similar to that for the other two types of lipoprotein.

Discussion. Several efforts have been made previously to study the serum lipoproteins with P₃₂ labelled phospholipids. Goldwater and associates(11) analyzed different levels of the ultracentrifuge cell for phospholipid radioactivity employing human sera at various times after feeding P₃₂. Differences in specific activity were found. Maurer(12) working primarily with rat serum separated the P₃₂ labelled serum fractions by filter paper electrophoresis. Considerable difficulty was encountered due to adsorption on the filter paper but labelling of the α type lipoproteins which predominate in the rat was noted. In the present study very little difference in

specific activity of the different serum lipoprotein fractions was observed. It was only in certain sera at early times after oral P₃₂ that slightly higher specific activities for the α_1 lipoproteins were encountered. These results are not surprising in view of the ready equilibration of tagged and untagged lipoproteins mixed *in vitro*. The small differences in specific activities may have resulted from variations in the ratios of lecithin, cephalin and sphingomyelin in the different lipoproteins. These various phospholipids are known to be differentially labelled in certain tissues (13). The high percentage of the total phospholipids of serum that is present as lecithin, approximately 75% (14,15), signifies that this substance is a constituent of both the α and β lipoproteins.

The exact mechanism of the *in vitro* exchange remains obscure. It appears to be similar to the striking interchange of labelled cholesterol between serum and red blood cells reported by Hagerman and Gould(16), and London and Schwarz(17) and seems to involve the phospholipid molecule. No definite conclusions were drawn in the latter reports as to the mechanism of this dynamic state. The possibility of an enzymatic mechanism is perhaps raised in the present experiments by the greater exchange on incubation at 37°C. than at 4°C. and by the decreased exchange with brief exposure to 60°C. However, no definitive evidence on this point was obtained and present knowledge of phospholipid synthesis(18) does not permit visualization of such a mechanism. The enzyme inhibitors tested had no effect on the reaction. The possibility that the exchange simply involved the phosphorus must be considered although there is little evidence in the literature suggesting such lability for phosphorus in the diester linkage of lecithin and cephalin. Experiments by Weinman and associates(19) have indicated a similar turnover rate for phospholipids injected as whole serum lipoproteins when either the P₃₂ or C₁₄ label was used. This would appear unlikely if the phosphorus showed special lability. In addition, Biggs(20) has recently observed interchange of tritium labelled cholesterol between rabbit lipoproteins *in vitro*. Eder(21) has also noted the exchange of P₃₂

in vitro in rabbit sera.

Summary. 1. Administration of P_{32} to humans by mouth resulted in labelling of the α_1 , α_2 and β lipoproteins separated by zone electrophoresis in a starch supporting medium. 2. Both phospholipid phosphorus and radioactivity of α_2 lipoprotein fraction increased after ordinary meals. 3. Specific activities of the different lipoproteins were very similar even at various time intervals following the oral radioactive phosphate. The only exception was a slightly higher specific activity for the α_1 lipoprotein in certain sera taken at early times. 4. *In vitro* experiments demonstrated transfer of phospholipid radioactivity from labelled β lipoproteins to unlabelled α_1 lipoproteins; also from labelled α_1 lipoproteins to unlabelled β lipoproteins.

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Leukemia VI. Effect of Amicetin on Transplanted Mouse Leukemia.*†

(21265)

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A new antibiotic, amicetin, isolated from *Streptomyces vinaceusdrappus* has been shown by DeBoer, Caron, and Hinman(1) to be active against *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Bacillus subtilis*. From the data of Hinman *et al.*(2) and Flynn

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† We wish to acknowledge gratefully the kindness of Eli Lilly and Co. and later the Upjohn Co. in providing us with supplies of Amicetin.

et al.(3) describing the isolation and purification of this compound and its chemical

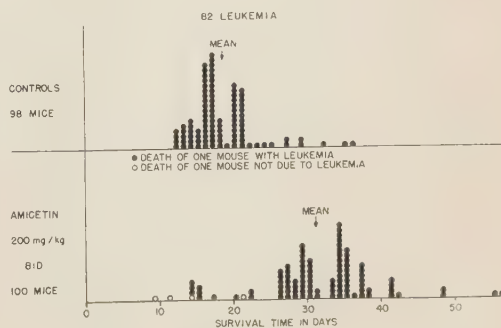


FIG. 1.

TABLE I. Comparative Effects of Amicetin, 6-Mercaptopurine, Azaserine, and Amethopterin against Transmitted Mouse Leukemia. 4 experiments.

Leukemia	Treatment	Dose, mg/kg	Dosage schedule	Route	No. mice	Survival time (days)	
						Mean	Range
82	Controls	—	—	—	10	19.7	17-32
	Amicetin	200	b.i.d.	i.p.	10	35.3	28-42
	"	2000	q.d.	p.o.	6	35.5	30-42
	Controls	—	—	—	9	14.9	13-16
	Amicetin	200	b.i.d.	i.p.	9	30.6	27-41
	Azaserine	20	t.i.w.	"	10	25.7	21-41
L1210	6-Mercaptopurine	100	t.i.w.	"	8	22.0	20-27
	Amethopterin	3	t.i.w.	"	9	17.4	17-20
	Controls	—	—	—	10	11.3	10-12
	Amicetin	200	b.i.d.	i.p.	9	11.1	9-12
	Azaserine	20	t.i.w.	"	9	14.9	12-17
	Amethopterin	3	t.i.w.	"	10	23.2	17-27
"	Controls	—	—	—	10	14.8	13-17
	Amicetin	200	b.i.d.	i.p.	10	15.4	14-20
	Azaserine	20	t.i.w.	"	10	19.7	16-21
	6-Mercaptopurine	50	t.i.w.	"	10	24.3	20-27

properties, it appears that one part of the molecule consists of an alpha-methyl-serine, a para-aminobenzoic acid and a cytosine moiety. The chemotherapeutic activity of this compound has been studied against various strains of mouse leukemia and the results are herewith reported.

Method. The technic for evaluation of the chemotherapeutic activity of a given drug by means of its ability to prolong the survival time of mice with transplanted leukemia has been described previously(4). In a typical experiment approximately one hundred mice of the F1 generation of the C58 ♂ x Bagg Albino ♀ cross were injected intraperitoneally with 0.1 cc of a saline suspension of leukemic spleen so diluted that 0.1 cc contained one million cells. Most of these studies were done on the 30th to 46th transplant generations of leukemia 82 which originated as a spontaneous leukemia in a C58 mouse in October, 1953. This transplanted leukemia kills in 12 to 20 days with an elevation of the white blood count to the 50,000 to 100,000 level and tremendous enlargement of liver and spleen, and some enlargement of lymph nodes. Line I(5) and Line I/A[†] in the same stock of mice and L1210 in dba mice were also studied. Twenty-four hours after inoculation these mice were divided into comparable groups of 10 mice each and treated intraperi-

toneally daily, twice daily or three times weekly for a 20-day period. The mice were observed for the development of leukemia and autopsied at death. If gross evidence of leukemia was not conclusive, microscopic sections were taken. The rationale behind the various steps of this technic has been outlined in previous reports by Burchenal *et al.*(6).

Results. In the scatter diagram in Fig. 1 the average survival time of control animals averaged approximately 18 days, whereas the mice injected intraperitoneally with amicetin at a dose of 200 mg/kg body weight twice daily for 5 days each week showed an average survival time of 31 days. Table I demonstrates the comparative effectiveness of amicetin against Leukemia 82 in comparison to such standard agents as amethopterin, 6-mercaptopurine and azaserine. Amicetin is more effective than either amethopterin, mercaptopurine or azaserine in this particular line of leukemia and is also active by mouth when given at a 5-fold increase in dosage. In Line I leukemia in the same mice, however, where both azaserine and amethopterin produce a high percentage of cures(7), amicetin does not significantly increase the survival time, and in the L1210 leukemia(8,9) where amethopterin, mercaptopurine and azaserine are effective in prolonging survival, amicetin is again without significant effect.

Discussion. Nothing is known at present as to the mechanism of action of amicetin in

[†] A variant of Line I made resistant to amethopterin by repeated passage through treated mice.

mouse leukemia, but it is conceivable that the cytosine moiety enables this compound to act as an antagonist of a precursor of nucleic acid or that the alpha-methyl-serine component acts as a serine antagonist. Studies in this laboratory have shown, however, that its anti-bacterial effect against *Streptococcus faecalis* and *L. arabinosus* cannot be prevented by cytosine, cytidine, cytidylic acid, uracil, uridine, or uridylic acid, serine or citrovorum factor. Like azaserine, amicetin is another agent derived from a filtrate of *Streptomyces* which is active in inhibiting the growth of mouse leukemia.

Summary. A new antibiotic, amicetin, isolated from *Streptomyces vinaceusdrappus* and known to contain a cytosine-paramino-benzoic acid and alpha-methyl-serine moiety has been shown to prolong survival time of mice with Line 82 leukemia. It has been without significant effect in two other strains of leukemia similarly studied.

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